Biochimie 132 (2017) 102–108

Contents lists available at ScienceDirect

Biochimie

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

Research paper

N-Linked glycans are an important component of the processive machinery of cellobiohydrolases

Alexander V. Gusakov [a, b, *], Anna S. Dotsenko [a, b], Aleksandra M. Rozhkova [a, b], Arkady P. Sinitsyn [a, b]

[a] Department of Chemistry, M. V. Lomonosov Moscow State University, Vorobyovy Gory 1/11, Moscow 119991, Russia
[b] Federal Research Centre "Fundamentals of Biotechnology", Russian Academy of Sciences, Leninsky Pr. 33, Moscow 119071, Russia

ARTICLE INFO

Article history:
Received 7 September 2016
Accepted 10 November 2016
Available online 14 November 2016

Keywords:
Cellobiohydrolase
Cloning
N-glycosylation
Processivity
Site-directed mutagenesis

ABSTRACT

Cellobiohydrolases (CBHs), belonging to glycoside hydrolase families 6 and 7 (GH6 and GH7), are the major components of cellulase systems of filamentous fungi involved in biodegradation of cellulose in nature. Previous studies demonstrated that *N*-linked glycans in the catalytic domains of GH7 CBHs significantly affect the enzyme activity against cellulosic substrates. The influence of *N*-linked glycans on the activity and processivity of recombinant GH6 CBH II from *Penicillium verruculosum* (PvCel6A) was studied using site-directed mutagenesis of the respective Asn residues. Depending on the position of *N*-glycans on the surface of a protein globule, they affected the enzyme activity against cellulose either negatively or positively. The decrease or increase in the degree of processivity of recombinant forms of PvCel6A generally correlated with activity changes against Avicel. The mechanism of the *N*-glycan influence seems to be universal for GH6 and GH7 CBHs. The observed effects for CBHs from both families are explained in terms of a mechanistic model that also makes clear our previously published data on the highly active CBH IIb from *Myceliophthora thermophila* (MtCel6B). This study, together with data of other researchers, strongly suggests that the *N*-linked glycans in the catalytic domains of GH6 and GH7 CBHs are involved in processive catalytic machinery of these enzymes. Data obtained should be taken into account during development of new and more effective biocatalysts by protein engineering techniques.

1. Introduction

Cellobiohydrolases (CBHs) are the major components of multienzyme cellulase systems of filamentous fungi involved in biodegradation of cellulose in nature [1,2]. Their content in a culture liquid may reach up to 80% of the total secreted protein [1]. Industrial cellulase preparations produced by fungal mutant strains recently found an application in the growing biotechnology of manufacturing second-generation biofuels from lignocellulosic residues [3]. So, much attention is focused now on finding cellulases with higher specific activity, better stability, lower susceptibility to inhibition, etc., as well as improving properties of already known enzymes using modern genetic engineering techniques [4–8].

CBHs are classified into families 6 and 7 of glycoside hydrolases [GH6 and GH7 in the Carbohydrate-Active Enzymes (CAZy) database]. They typically have a bimodular structure, consisting of a catalytic domain (CD) and a cellulose-binding module (CBM), which are connected by a flexible peptide linker [2,9]. The active site of CBHs represents a kind of tunnel formed by peptide loops. CBHs of type I (CBH I, EC 3.2.1.176), belonging to the GH7 family, initiate their attack on cellulose from reducing ends of a polymeric chain, and they sequentially remove cellobiose residues via a processive mechanism. The GH6 CBHs of type II (CBH II, EC 3.2.1.91) primarily act on cellulose molecules from the nonreducing

Abbreviations: CBH, cellobiohydrolase; CBM, cellulose-binding module; CD, catalytic domain; GH6 and GH7, glycoside hydrolase families 6 and 7; MS, mass spectrometry; MtCel6B, *Myceliophthora thermophila* family 6 cellobiohydrolase IIb; PCR, polymerase chain reaction; PfCel7A, *Penicillium funiculosum* family 7 cellobiohydrolase I; PvCel6A, *Penicillium verruculosum* family 6 cellobiohydrolase II; PvCel7A, *Penicillium verruculosum* family 7 cellobiohydrolase I; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TrCel7A, *Trichoderma reesei* family 7 cellobiohydrolase I.

* Corresponding author. Department of Chemistry, M. V. Lomonosov Moscow State University, Vorobyovy Gory 1/11, Moscow 119991, Russia.
E-mail address: avgusakov@enzyme.chem.msu.ru (A.V. Gusakov).

http://dx.doi.org/10.1016/j.biochi.2016.11.004
0300-9084/

ends [1,2]. Compared to the GH7, the GH6 CBHs are generally characterized by a lower degree of processivity because of a shorter and more opened active site tunnel [1,2,10].

Like other fungal enzymes, CBHs are usually glycosylated. As a rule, the *N*-glycosylation is a characteristic of catalytic domains, while peptide linkers, rich in Ser and Thr residues, are heavily decorated with *O*-linked glycans [9,11,12]. The roles of *N*- and *O*-glycosylation in the structure and function of cellulases were not well understood until recent years. It has been thought that both types of glycans may participate in correct folding of a protein and maintaining its stability, while the *O*-linked glycans in a linker may protect it against protease action [11,12]. Jeoh and co-workers reported that an over-*N*-glycosylated recombinant CBH I of *Trichoderma reesei* (rTrCel7A) expressed in *Aspergillus niger* displays a reduced activity on cellulose relative to the native enzyme [13]. Then the same team of researchers showed that eliminating an existing *N*-glycosylation site in rTrCel7A or introducing a new *N*-glycosylation site in recombinant Cel7A of *Penicillium funiculosum* (rPfCel7A) by site-directed mutagenesis leads to an increased enzyme activity against crystalline cellulose [14]. We successfully applied this approach for boosting the specific activity of recombinant CBH I from *Penicillium verruculosum* (rPvCel7A expressed in *Penicillium canescens*) and showed that the *N*-linked glycans are involved in complex interactions with a polymeric substrate in catalysis [15].

In this paper, we demonstrate that the *N*-linked glycans in the CD of recombinant *P. verruculosum* CBH II (rPvCel6A) represent an important part of the enzyme processive catalytic machinery. These data, together our previous data on the highly active CBH IIb (MtCel6B) from *Myceliophthora thermophila* (formerly *Chrysosporium lucknowense*) [16], indicate that the mechanism of the *N*-glycan influence seems to be universal both for GH6 and GH7 processive CBHs.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis of PvCel6A

The *cbh2* gene encoding *P. verruculosum* CBH II (PvCel6A) was cloned into *P. canescens* PCA10 (*niaD*-) strain that represents an efficient fungal host for expression of heterologous proteins [15,17]. Four pairs of primers were constructed to conduct the N219A, N265A, N279A and N395A substitutions in the recombinant PvCel6A (Table S1). Cloning the wild type enzyme and its site-directed mutagenesis was carried out using the same methodological approaches as reported previously for PvCel7A and PvCel5A [15,18,19]. In brief, the full-size *cbh2* gene and its mutated forms were cloned into a linear PC1 shuttle vector [17] and the resulting expression constructs were transformed into *Escherichia coli* MachI cells for the production and analysis of DNA material. After confirmation of the gene sequences by sequencing, the expression constructs were transformed into *P. canescens* by the method of PEG-mediated protoplast fusion [20]. After screening the transformants, the best selected recombinant strains, containing the target genes, were cultured in 3-L fermenters KF-104/3 (Prointex, Russia) as described elsewhere [15].

Standard reagents, such as Long Polymerase mix, Pfu polymerase, Phire polymerase and other components supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA), were used for polymerase chain reaction (PCR). Isolation of genomic DNA, isolation of amplified parts of the *cbh2* gene from agarose gel, isolation of plasmid DNA, as well as purification of the PCR products, were carried out using QIAGEN Kits (QIAGEN, Valencia, CA, USA).

2.2. Enzyme purification

Purification of the native PvCel6A and its recombinant forms was carried out essentially as described elsewhere [21]. In brief, the purification steps included preliminary desalting of the crude enzyme preparations on a Bio-Gel P-2 column (Bio-Rad Laboratories, Hercules, CA, USA), anion-exchange chromatography on a Source 15Q column followed by hydrophobic interaction chromatography on a Source 15 Isopropyl column (Pharmacia, Uppsala, Sweden). Enzyme purity was assayed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, Hercules, CA, USA). Staining of protein bands was carried out with Coomassie Blue R-250 (Ferak, Berlin, Germany). The results of SDS-PAGE are shown in Fig. 1. Protein concentration in samples was determined by the modified Lowry method [22], using bovine serum albumin as the standard.

2.3. MALDI-TOF mass spectrometry analysis

Pieces of protein bands, corresponding to the enzymes under study, were excised from the gels after the SDS-PAGE and then digested with pepsin of sequencing grade (Sigma, St. Louis, MO, USA) using a standard protocol [23]. The resulting peptides were extracted from the gel with 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry (MS) on an UltrafleXtreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). FindPept and GlycoMod tools (http://www.expasy.org/tools/#proteome) were used for peptide and glycopeptide identification as described elsewhere [24,25].

2.4. 3D structure modeling

3D models of PvCel6A and MtCel6B CDs were built using SWISS-MODEL protein structure homology-modeling server at the ExPASy

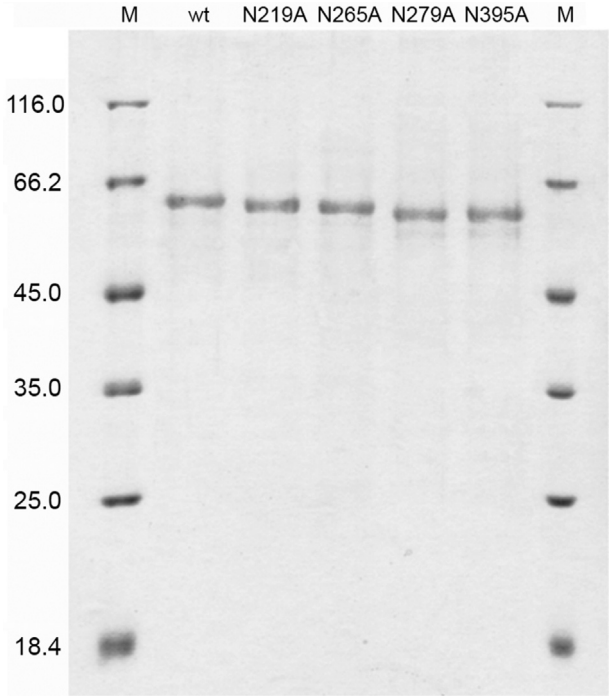

Fig. 1. SDS-PAGE of rPvCel6A of wild type (wt) and its mutant forms. Left and right lanes represent molecular markers (in kDa).