Biochimie 110 (2015) 45–51

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Glucoamylases from *Penicillium verruculosum* and *Myceliophthora thermophila*: Analysis of differences in activity against polymeric substrates based on 3D model structures of the intact enzymes

Pavel V. Volkov [a], Aleksandra M. Rozhkova [a, *], Alexander V. Gusakov [a, b], Ivan N. Zorov [a, b], Arkady P. Sinitsyn [a, b]

[a] A.N. Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky Pr. 33, Moscow 119071, Russia
[b] Department of Chemistry, M.V. Lomonosov Moscow State University, Vorobyovy Gory 1/11, Moscow 119991, Russia

ARTICLE INFO

Article history:
Received 6 November 2014
Accepted 12 December 2014
Available online 23 December 2014

Keywords:
Glucoamylase
Modeling
Myceliophthora thermophila
Penicillium verruculosum
Hydrolysis

ABSTRACT

Two glucoamylases, a recombinant enzyme from *Penicillium verruculosum* (PvGla) heterologously expressed in *Penicillium canescens* RN3-11-7 (*niaD-*) strain and a native glucoamylase from *Myceliophthora thermophila* (MtGla), were purified and their properties were studied. MtGla displayed 2–5-fold higher specific activities against soluble starch, amylose and amylopectin than PvGla. MtGla also provided higher glucose yields in extended hydrolysis of the polymeric substrates. Analysis of 3D model structures of the intact PvGla and MtGla, which were built using the 2vn7.pdb crystal structure of the intact *Trichoderma reesei* glucoamylase (TrGla) as a template, showed that the reason for lower hydrolytic performance of PvGla in comparison to MtGla may be less strong interactions between the enzyme domains as well as a longer (by 17 residues) linker in the first enzyme.

1. Introduction

Glucoamylases (glucan 1,4-α-glucosidases, EC 3.2.1.3) catalyze a successive removal of 1,4-linked α-D-glucose residues from the non-reducing ends of polymeric α-D-glucans or maltooligosaccharides with release of β-D-glucose via inverting mechanism [1,2]. Some glucoamylases can hydrolyze also 1,6-α-D-glucosidic bonds when the next bond in the sequence is 1,4-α-D-glucosidic. Starch, amylose, amylopectin, pullulan and maltooligosaccharides are natural substrates for glucoamylases. Most glucoamylases belong to the glycoside hydrolase family 15 (GH15) (http://www.cazy.org/GH15_all.html) [3]. A fewer glucoamylases from bacteria or archaea belong to the GH97 family.

Glucoamylases are widely abundant in nature, and most of them are of microbial origin. Fungal glucoamylases produced by *Aspergilli* (*A. awamori* and *A. niger*) found commercial industrial applications. In combination with α-amylases or glucose isomerases, they are used for production of glucose or glucose-fructose syrups [4–6]. Glucose, as the final product of enzymatic hydrolysis of starch, is widely used in food and alcoholic beverage industries, for obtaining products of microbiological synthesis, and for production of the first-generation biofuels [2,4,5,7].

In this paper, we compared hydrolytic performance of two purified fungal glucoamylases from *Penicillium verruculosum* and *Myceliophthora thermophila* (PvGla and MtGla, respectively) in hydrolysis of different natural polymeric substrates, and based on the alignment of enzyme amino acid sequences and analysis of their 3D models we analyzed possible reasons for differences in the catalytic efficiency of PvGla and MtGla. Some properties of the recombinant 88 kDa PvGla expressed in *Penicillium canescens* RN3-11-7 (*niaD-*) strain have been described previously (without providing sequence information) [8], while those for the MtGla are reported for the first time here.

2. Materials and methods

2.1. Fungal strains and fermentation media

Strains and plasmids used for cloning and expression of the *gla* gene encoding *P. verruculosum* glucoamylase were described

Abbreviations: CD, catalytic domain; MtGla, *Myceliophthora thermophila* glucoamylase; PCR, polymerase chain reaction; PvGla, *Penicillium verruculosum* glucoamylase; SBD, starch-binding domain; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TrGla, *Trichoderma reesei* glucoamylase.

* Corresponding author. Tel.: +7 910 4644791; fax: +7 495 9542732.
E-mail address: amrojkova@mail.ru (A.M. Rozhkova).

http://dx.doi.org/10.1016/j.biochi.2014.12.010
0300-9084/

elsewhere [8,9]. The mutant *P. canescens* RN3-11-7 strain (VKPM F-436), deficient in the nitrate reductase gene (*niaD*-), was used as a host strain for transformation and chromosomal DNA preparation. *M. thermophila* ATCC 42464 strain was used for obtaining an enzyme preparation.

A medium for cultivating *M. thermophila* contained 24 g/L wheat meal, pretreated with a thermostable amylase. Culture conditions for *P. canescens* were described elsewhere [8]. Fungi were cultured in a 3 L bioreactor KF-104/3 (Prointex, Russia), the initial pH in the liquid medium was adjusted to 4.8–5.0 with 10% HCl.

2.2. Construction of the fungal expression plasmid and generation of mutants

The nucleotide sequence corresponding to the full-length *gla* gene was amplified by polymerase chain reaction (PCR), wherein a genomic DNA of *P. verruculosum* WA30 was used as a matrix. The genomic DNA was isolated using DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). In order to amplify the *gla* sequence, the pGA_UpLIC and pGA_LowLIC primers were used:

pGA_UpLIC: gca cag gca gca gga gct gcc cct cag ctg tct cct cgt gct aca ac
pGA_LowLIC: aga gca agc cga gca ggt cta tcg cca agt atc ctc aga agc tgt gct

PCR was performed on a MyCycler equipment (Bio-Rad Laboratories, Hercules, CA, USA) according to the following protocol: 5 min at 95 °C, followed by 20 cycles of 1.5 min at 95 °C, 2 min at 50 °C, 2 min at 68 °C, and then 20 cycles of 10 min at 68 °C, 10 min at 4 °C. The resulting PCR product was purified from agarose gel using QiAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and then it was cloned into the pXEG linearized vector using a ligation - independent method [10].

Briefly, the PCR product (2096 bp) and the pXEG linearized vector were treated with T4 DNA polymerase (Thermo Fisher Scientific Inc., Waltman, MA, USA) in the presence dATP and dTTP (Thermo Fisher Scientific Inc., Waltman, MA, USA), respectively. Cured insert was ligated into the pXEG vector by mixing 50 ng of vector with 150 ng of insert. The mixture was incubated for 30 min at 22 °C and then transformed into *E. coli* MachI (Invitrogene, Carlsbad, CA, USA) using a standard transformation protocol [11]. Thus, a plasmid construct pPrXylA-GA, containing the gene encoding PvGla, was obtained. The absence of mutations in the *gla* gene was confirmed by its sequencing in both directions by the method described by Sanger et al. [12].

The pPrXylA-GA plasmid construct was directed into protoplasts of the recipient RN3-11-7 (*niaD*-) strain together with a plasmid pSTA10 (10:1, μg), using the modified method described by Aleksenko et al. [13]. The pSTA10 plasmid, used for transformation, carried a nitrate reductase gene providing complementation of a defective *niaD* gene in the recipient strain. This allowed selection of the transformants on minimal media with $NaNO_3$. Transformation efficiencies typically reach hundreds of transformants per μg of transforming DNA, with co-transformation frequencies of 80% and higher [13].

2.3. Enzyme purification

PvGla was purified from the culture filtrate as described elsewhere [8].

For isolation of MtGla, proteins contained in the crude enzyme preparation were preliminary precipitated with ammonium sulfate (80% saturation at 25 °C) followed by a desalting procedure on a Bio-Gel P-4 (Bio-Rad Laboratories, Hercules, CA, USA) with the use of 0.02 M bis-Tris/HCl buffer, pH 6.8. Enzyme purification was carried out by anion-exchange chromatography on a Source 15Q HR 16/5 column (Pharmacia, Uppsala, Sweden). A sample containing 10 mg of protein was applied on the column equilibrated with 0.02 M bis-Tris/HCl buffer, pH 6.8. The bound protein was eluted with a gradient of 0–0.75 M NaCl at a flow rate of 1 mL/min (60 mL total volume). Buffer replacement in collected fractions was performed on a Bio-Gel P-4 column (Bio-Rad Laboratories, Hercules, CA, USA).

The enzyme purity was characterized by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrofocusing. SDS-PAGE was carried out in 12% gel using a Mini Protean II equipment (Bio-Rad Laboratories, Hercules, CA, USA). Isoelectrofocusing was performed on a Model 111 Mini IEF Cell (Bio-Rad Laboratories, Hercules, CA, USA). Staining of protein bands was carried out with Coomassie Blue R-250 (Ferak, Berlin, Germany).

Protein concentration in samples was determined by the modified Lowry method [14], using bovine serum albumin as the standard, or by absorbance at 280 nm.

2.4. MALDI-TOF mass spectrometry peptide fingerprinting

The in-gel tryptic digestion of protein bands after the SDS-PAGE was carried out essentially as described by Smith [15]. Trypsin (Promega, Madison, WI, USA, modified, 5 μg/mL) in 50 mM NH_4HCO_3 was used for a protein digestion. The resulting peptides were extracted from a gel with 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry on Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Peptide and enzyme identification was carried out using Mascot peptide mass fingerprint server (http://www.matrixscience.com/http://www.matrixscience.com/) as well as FindPept and GlycoMod tools (http://www.expasy.org/tools/#proteome) as described elsewhere [16].

2.5. Enzyme 3D structure modeling

3D structure models of PvGla and MtGla were built using an automated mode of SWISS-MODEL protein structure homology-modeling server, accessible via the ExPASy web server (http://swissmodel.expasy.org/) [17,18]. The 1gah.pdb and 2vn7.pdb crystallographic structures of the glucoamylases from *A. awamori* and *Trichoderma reesei*, sharing 56–70% identity to the enzymes under study, were used as templates in both cases.

2.6. Enzyme activity assays

Glucoamylase activity was determined by analyzing reducing sugars released after 10 min of enzyme reaction with 5 mg/mL soluble starch from potato (Reakhim, Russia), amylose (Sigma, St. Louis, MO, USA) and amylopectin (Serva Electrophoresis GmbH, Heidelberg, Germany) at pH 4.7 (0.05 M Na-acetate buffer) and 30 °C [8]. Reducing sugars were analyzed by the Nelson–Somogyi method [19,20]. Enzyme activity against *p*-NP-α-glucopyranoside (Sigma, St. Louis, MO, USA) were determined at pH 5.0 and 40 °C as described elsewhere [21]. Activity against maltose was determined at pH 4.7 and 40 °C as described elsewhere [22].

Enzyme activities were expressed in international units. One unit of activity corresponded to the quantity of enzyme hydrolyzing 1 μmol of substrate or releasing 1 μmol of reducing sugars (in glucose equivalents) per minute.

Study of effect of pH on the glucoamylase activity was carried out using soluble starch (Reakhim, Russia) as a substrate. The activity assays were performed as described above, except 0.1 M