Carbohydrate Research 499 (2021) 108211

Contents lists available at ScienceDirect

Carbohydrate Research

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

Properties of recombinant endo-β-1,6-glucanase from *Trichoderma harzianum* and its application in the pustulan hydrolysis

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ARTICLE INFO

Keywords:
Pustulan
Pustulanase
GH5
Gentiobiose
Penicillium verruculosum
Hydrolysis

ABSTRACT

The gene encoding *Trichoderma harzianum* fungus pustulanase (ThBGL1.6, GH5 family, endo-β-1,6-glucanase, EC 3.2.1.75) was cloned and heterologously expressed by the highly productive *Penicillium verruculosum* fungus. The recombinant ThBGL1.6 was purified and its properties were studied. The ThBGL1.6 had an observed molecular mass of 46 kDa (SDS-PAGE data) and displayed maximum of the enzyme activity at pH 5.0 and 50 °C. At 45 °C, the ThBGL1.6 was stable for at least 3 h. The K_m was 1.0 g/L with pustulan as the substrate. Reaction product analysis by HPLC clearly indicated that ThBGL1.6 has an endo-hydrolytic mode of action against pustulan as specific substrate. It was also identified that gentiobiose is the main reaction product at studying of long-term pustulan hydrolysis.

1. Introduction

β-Glucan is a widespread polymer obtained from oats, barley, bacteria, yeast, algae, and mushrooms [1]. It consists of glucose units with different chemical bonds. Noncellulosic β-glucans, for example, barley glucan (β-1,3/1,4)-glucan), laminarin, schizophylan, epiglucan and scleroglucan have a complicated structure, with a diversity of linkage types in either linear or branched chains [2]. Curdlan and paramylon (β-1,3-glucans) have relatively simple structures, consisting of a single linkage type in an unbranched chain [3]. The insufficiently studied type of β-glucan is pustulan (β-1,6-glucan). In fact, only a few linear β-1,6-glucans have been identified, the best known being pustulan from *Umbilicaria* species [4] and lutean from *Penicillium luteum* [5]. β-1,6-linkages occur frequently in the glucans of yeast and filamentous fungal cell walls [6,7] and may be part of the biofilms that are formed by microorganisms on their surface. For example, β-1,6-glucan has been shown to be an important component of the *Saccharomyces* cerevisiae, Candida albicans, Cryptococcus neoformans cell wall [8–10]. Previously, it was discovered that β-1,6-glucan in S. cerevisiae has been shown to form cross-links with β-1,3-glucan, chitin, and with a glycosylphosphatidylinositol GPI anchor oligosaccharide [11]. Thus, the β-1,6-glucan plays an important role in the formation of the microorganisms cell wall matrix [11]. Pustulan can be a source of enzymatic mono- and oligosaccharides with different functional properties [12].

Pustulanase, also known as β-1,6-glucanase (EC 3.2.1.75) is a key enzyme, which catalyzes the pustulan hydrolysis by the endo-depolymerase mechanism with the release of oligosaccharides and glucose [5]. According to the CAZy database (http://www.cazy.org/) [13], almost all known β-1,6-glucanases are classified into families 5 and 30 of glycoside hydrolases (GH5 and GH30). Although, β-1,6-glucanases are widely distributed among fungi, few of them have been purified and characterized [5,14–16].

The present paper describes the cloning of *bgl1.6* gene from *Trichoderma harzianum* and its high level expression in *Penicillium verruculosum* [17] recipient strain, purification, properties and the mode of action of pustulanase on pustulan. Heterologously expressed pustulanase is required for hydrolysis of polysaccharides which are part of the lichens cell wall: β-glucans, α-glucans, and galactomannans [18].

Previously, we developed expression system to produce homologous and heterologous enzymes in a high-cellulase fungal *P. verruculosum* B1-

Abbreviations: MCC, microcrystalline cellulose; Ge, gentiobiose; ThBGL1.6, endo-β-1,6 – glucanase from *Trichoderma harzianum*; MWD, molecular weight distribution; rAnBGL, recombinant *Aspergillus niger* β-glucosidase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; pNPG, *p*-nitrophenyl-β-D-glucopyranoside; CMC, carboxymethyl cellulose sodium salt.

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https://doi.org/10.1016/j.carres.2020.108211
Received 29 June 2020; Received in revised form 21 November 2020; Accepted 27 November 2020
Available online 1 December 2020

537 host. It is based on a homologous inducible *cbhI* promoter [17,19]. Productivity of the recipient strain on standard growth medium reaches up to 50 g of extracellular protein per 1 L of culture fluid.

2. Experimental

2.1. Microbial strains and media

The mycelium of the *T. harzianum* strain was isolated from the surface of a crumbling tree and grown on a medium contained (in g/L): glucose – 5.0, yeast extract – 10.0 and potassium phosphate – 25.0. The genomic DNA from *T. harzianum* mycelia was isolated using DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). Auxotrophic *P. verruculosum* B1-537 strain with a knock-out nitrate reductase gene (*ΔniaD*) [19,20] was used as a host strain in transformation. The *Escherichia coli* MachI™T1R strain (Invitrogen, Carlsbad, CA, USA) was applied for bacterial transformation and isolation of plasmids containing target genes.

A medium for cultivation of *P. verruculosum* transformants contained (in g/L): microcrystalline cellulose (MCC) – 40; yeast extract – 10; KH_2PO_4 – 15; $(NH_4)_2SO_4$ – 5; $MgSO_4 \cdot 7H_2O$ – 0.3; $CaCl_2 \cdot 2H_2O$ – 0.3. *P. verruculosum* B1-537 (*ΔniaD*) was used as control.

The composition of the medium for *P. verruculosum* fermentation was the following (in g/L): MCC – 60; glucose – 40; wheat bran – 10; yeast extract – 10; KH_2PO_4 – 7; $(NH_4)_2SO_4$ – 5; $MgSO_4 \cdot 7H_2O$ – 0.3; $CaCl_2 \cdot 2H_2O$ – 0.3 (pH 4.5–5.0, 32°C, fermentation time 144 h).

2.2. Construction of the expression plasmids and transformation of P. verruculosum

Plasmid construction and production of recombinant strains was carried out as described previously [19–21]. Primers were designed based on the homology of genes encoding similar β-glucanases from NCBI database: CAA55789.1, XP_024773174.1 and OPB46546.1. Briefly, using the freshly isolated genomic DNA from *T. harzianum* and two pairs of primers shown below, the 1352 bp PCR-product was amplified by polymerase chain reaction (PCR) using a MyCycler equipment (Bio-Rad Laboratories, Hercules, CA, USA):

Pust-LIC5 5′- caa-aca-gaa-gca-acc-gac-aca-atg-aag-tac-tcc-atc-gtt-gct-ccg-g - 3’.

Pust-LIC3 5′- gag-gag-aag-cc-ggt-tac-ctg-aat-cca-gcg-cag-aca-tcc-tgg-t - 3’.

Then, using the LIC-cloning method [22], the *bgl1.6* gene was cloned into the pCBHI vector under control of *cbhI* promoter encoding homologous cellobiohydrolase I [23]. Thus, pCBHI-Pust was obtained (Fig. S1). Protoplasts of *P. verruculosum* B1-537 host strain were transformed by pCBHI-Pust plasmid together with the pSTA10 co-transforming plasmid at the ratio of 6:1 μg using the transformation protocol [24]. The pSTA10 plasmid contained a homologous nitrate reductase (*niaD*) gene, allowing the selection of the resulting transformants on medium supplemented with $NaNO_3$ (10 mM). The transformation frequency was 20–40 clones per 1 μg of pCBHI-Pust plasmid, which corresponds to the literature data for *Penicillium* strains [24].

2.3. Screening and cultivation of transformants

Screening of transformants was carried out in glass tubes (total volume 50 mL, fermentation medium volume 10 mL). As a result of primary screening, several clones of *P. verruculosum* (PV4, PV15, PV35 and PV44) with a high activity of the culture filtrates against pustulan were selected.

The best selected clones were cultivated in 3-L glass bioreactors KF-104/3 (Prointex, Moscow, Russia). Fermentation was carried out in fed-batch mode with glucose addition (every 12 h after 2 days), three additions of MCC and one addition of salts.

After completion of cultivation in fermenters, the culture broth was centrifuged at 4000 rpm for 20 min on a centrifuge Avanti JXN-26 (Beckman Coulter, Atlanta, GA, USA) to remove biomass and insoluble components of the nutrient medium. The supernatant was freeze-dried on a VirTisBenchTop 2K ES freeze dryer (SP Scientific, Warminster, PA, USA).

2.4. Enzyme purification

Desalting and fractionation of crude freeze-dried enzyme preparations were carried out on AKTA Purifier system (GE Healthcare, Sweden). Proteins were preliminary precipitated with ammonium sulfate (80% of saturation) followed by a desalting on a Bio-Gel P-4 column (Bio-Rad Laboratories, Hercules, CA, USA) equilibrated by 0.01 M Bis-Tris/HCl buffer, pH 6.8. The desalted protein solution was fractionated by anion-exchange chromatography (AEC) on a Source 15Q column (Pharmacia, Uppsala, Sweden) in Bis-Tris/HCl starting buffer (pH 6.8, 0.01 M), the bound proteins were eluted with a gradient of NaCl (0–0.4 M). Protein fractions with pustulanase activity were purified by hydrophobic interaction chromatography (HIC) on a Source 15ISO column (Pharmacia, Uppsala, Sweden). Ammonium sulfate was added to the sample to give 1.7 M $(NH_4)_2SO_4$ in 50 mM Na-acetate buffer, pH 5.0, the elution of proteins was performed in a reversed gradient of $(NH_4)_2SO_4$ from 1.7 to 0 M. The fraction with a highest pustulanase activity was selected for the final purification by gel filtration on a Superose 12 column (GE Healthcare, UK). Elution was performed in the isocratic mode in 0.01 M Na-acetate buffer, pH 5.0.

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

Protein concentration in samples was determined by the modified method of Lowry et al. [25], using bovine serum albumin as the standard.

2.5. Enzyme identification by MALDI-TOF mass spectrometry

Identification of the pustulanase was carried out by MALDI-TOF mass spectrometry (MS) peptide fingerprinting of trypsin-digested proteins from the bands after IEF as described earlier [23,26]. MALDI-TOF MS of peptides was carried out on an ultrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Peptides in the mass spectra were identified using the online service FindPept (https://web.expasy.org/findpept/). They were compared with the peptides obtained by theoretical trypsinolysis of the bgl 1.6 gene amino acid sequence of *T. harzianum* pustulanase. Glycopeptides and structures of N-linked glycans were identified using the GlycoMod tool (https://web.expasy.org/glycomod/).

2.6. Enzyme activity assays

Enzyme activity against polymeric substrates was assayed using the modified Nelson-Somogyi method [27] of determination of the reducing sugars (RS) released from pustulan (InvivoGen, France), laminarin (Sigma, St. Louis, MO, USA), β-glucan (Megazyme), Na-salt carboximetylcellulose (CMC, Sigma, St. Louis, MO, USA) and gentiobiose (Megazyme). A solution of the substrate (10 g/L) in 0.1 M Na acetate buffer, pH 5.0, was incubated with the enzyme at 50 °C for 10 min (reaction volume 0.2 mL), and the reaction was stopped by adding 0.2 ml of the Somogyi reagent. After incubation of the obtained solution for 40 min in a boiling water bath, 0.2 ml of the Nelson reagent was added. The resulting solution was cooled in cold water for 10 min and 0.4 mL of acetone and 1 mL of distilled water were added. After centrifugation of the sample for 2 min at 13,000 rpm, the absorbance of the supernatant at

610 nm was measured against a substrate blank of the same concentration (without added enzyme). Glucose (Sigma, St. Louis, MO, USA) was used for preparing a calibration plot.

Enzyme activities were expressed in international units. One unit of activity corresponded to the quantity of enzyme converting 1 μmol of substrate or releasing 1 μmol of RS per minute. All activity assays were performed in triplicates.

During pH detection, the activity assays were carried out as described above, except 0.1 M citrate–phosphate universal buffer was used for maintaining the necessary pH in the reaction system instead of the acetate buffer.

2.7. Determination of kinetic parameters

The kinetic parameters of purified pustulanase were determined at 50 °C and pH 5.0 (0.1 M Na-acetate buffer) using pustulan (InvivoGen, France) as a substrate. The substrate concentration was varied in the range of 0.1–3.0 g/L. The initial rates of the enzymatic reaction were determined by the Nelson-Somogyi method [27] as described above, and then the values of K_m and V_{max} were calculated by analyzing data obtained by the method of nonlinear regression using the OriginPro 8 software. The experiments were conducted in triplicates.

2.8. Enzyme thermostability studies

The solutions of purified recombinant pustulanase were incubated at pH 5.0 (0.1 M Na-acetate buffer) and 40, 45, 50, 55 or 60 °C in a thermostat TS-100 (Biosan SIA, Latvia). After a certain incubation time, aliquots of the solution were taken, immediately cooled down, and the residual activity against pustulan was determined under the standard conditions (see above). The initial activity of the enzyme sample was taken as 100%. The experiments were carried out in triplicates.

2.9. Progress kinetics of hydrolysis

Hydrolysis of pustulan (5 mg/mL) and lichen (100 g/L) was carried out for 48 h at 40 °C and pH 5.0 (0.1 M Na-acetate buffer). Purified pustulanase as well as crude pustulanase enzyme preparations were used for hydrolysis. The pustulanase dosage was 0.1–1.0 units of activity per 1 mL (1–10 U/g) of pustulan solution. Aliquots were taken from the reaction mixture at a certain time, boiled at 100 °C water bath for 5 min to terminate the reaction and centrifuged to remove the denatured protein. The amount of glucose released was determined by the glucose oxidase/peroxidase assay [28] using Photoglucose kit (Impact Ltd., Russia).

Agilent 1200 HPLC system (Agilent Technol. Inc., USA) with a Coulochem III electrochemical detector (ESA/Thermo Fisher Scientific Inc., USA) was used for the hydrolysis products analysis. The samples were dissolved in 100 mM NaOH solution and applied on a CarboPac PA-100 column (Thermo Fisher Scientific Inc., USA). An isocratic elution in 100 mM NaOH was used for the first 5 min, followed by linear gradient elution up to 450 mM of Na-acetate in 30 min. Detection was carried out in pulsing amperometry mode (+100 mV for 400 ms, −1500 mV for 20 ms, +600 mV for 30 ms, −100 mV for 50 ms), calibration was performed using glucose, cellobiose, gentiobiose (Ge) and cellooligosacharides (Sigma, MO, USA) as the standards.

3. Results and discussion

3.1. Pustulanase (ThBGL1.6) sequence analysis

PCR fragment amplified from gDNA *T. harzianum* consists of 1352 bp. The coding part of the gene (1290 bp) encodes a polypeptide of 430 amino acid residues (GenBank accession number MT362713). Analysis of the sequence using software package on the ExPASy Proteomic Server (http://ca.expasy.org/tools/dna.html) and comparison of nucleotide and amino acid sequences with those for other enzymes belonging to the same protein family showed the presence of a single intron (63 bp) in the *bgl1.6* gene.

The amino acid sequence for *T. harzianum* ThBGL1.6, translated from the gene sequence, is shown in Fig. 1. BLAST analysis showed that the enzyme displays similarity to GH5 endo-β-1,6-glucanases. So, it was classified as glycoside hydrolase family 5 (GH5, pustulanase, ThBGL1.6) (http://www.cazy.org/GH5_characterized.html). This protein sequence has homology with other β-glucanases, but the highest sequence identity was observed in the case of endo-β-1,6-glucanases from *T. lixii* (99.07%, AN: CAA55789.1), *T. harzianum* (99.30%, AN: XP_024773174.1) and *T. guizhouense* (98.84%, AN: OPB46546.1). A putative catalytic structure IEVLNEP was found inside the amino acid sequence of the ThBGL1.6. Moreover, this active site is independent of the endo- or exo-type of hydrolysis of the glucan, because it is conserved in exo-as well as endo-enzymes [29].

In accordance to SignalP tool prediction (http://www.cbs.dtu.dk/services/SignalP) the first 17 amino acid residues of the ThBGL1.6 (Fig. 1) are assumed to be a signal peptide.The theoretical molecular mass and pI of the mature protein calculated from its amino acid composition without signal peptide by the Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan) were 46315 Da and 5.36, respectively. A single potential N-glycosylation site was discriminated in ThBGL1.6: Asn12, however, the presence of protein glycosylation on the protein surface was not confirmed (it's shown in box in Fig. 1).

3.2. Homologous expression of ThBGL1.6

The *T. harzianum bgl1.6* gene was cloned and heterologously expressed into *P. verruculosum* B1-537 host strain. The corresponding recombinant enzyme is referred here as ThBGL1.6. Several clones of *P. verruculosum* (4, 15, 35 and 44) with a highest activity of the culture filtrates against pustulan were selected after screening of the transformants in shake flasks. The SDS-PAGE analysis of the crude enzyme preparations (Fig. S2) showed that new protein band with a mass of ~46 kDa appeared in culture filtrates produced by new recombinant strains of *P. verruculosum,* carrying the *bgl1.6* gene, compared with the control sample based on the initial recipient *P. verruculosum* strain.

Specific activities of the dry enzyme preparations against different substrates are shown in Table 1. The highest specific activity against pustulan was observed for clone 15 (PV15, 80 U/mg of protein). The specific activity of the PV15 containing ThBGL1.6 is increased by 400 times in comparison with the activity of the enzyme preparation (EP) obtained from the control *P. verruculosum* host strain. It should be noted that the *P. verruculosum* recipient strain (PV-control) displayed only a trace level of pustulanase activity (0.2 U/mg), that is why this recipient strain was selected for heterologous pustulanase expression. The specific activity of the PV-control enzyme preparation against MCC was 0.6 U/mg, while it decreased from 16 to 41% in the series of PV samples containing the recombinant ThBGL1.6. This phenomenon is related with the use of the *cbh1* gene promoter of the respective recipient strain for the heterologous expression of the recombinant BGL1.6 under study, resulting in a lower expression of cellobiohydrolase I by the new recombinant strains of *P. verruculosum* as we have previously demonstrated [30].

PV 4, PV15, PV35 and PV44 transformants were selected for cultivation in a 3-L laboratory fermenter, and the PV15 dry EP obtained was used for isolation and purification of ThBGL1.6. The results of pustulanase activity and protein increase during the growth of the best strain are presented in Table S1. The pustulanase activity in culture fluid at the end of best producer (PV 15) cultivation was 1360 U/mL.

3.3. Enzyme purification

Purification of the ThBGL1.6 was carried out using three stages of chromatography. Enzyme sample, obtained after the removal of non-

```
  1 mkysivapailagtafaWLPQDRDLKAFNQTARFEQLGKRFEPALASGIT 50
 51 KIRGVNFGGWLISEPWMMSNEWNNNMGCNGAASEFDCMRNNYMGSKRATG 100
101 NTKFQNHYRDWINPATVQSVHDVGLNTIRIPIGYWSYNAIVDTASEPFAD 150
151 GNLQLPYLDAVVQKAADLGICVIIDLHGAPGGQQQDAFTGQNPNPAGFYN 200
201 SYDYGRAEKWLSWMTNRIHTNPAYSTVGMIEVLNEPVSRHDGGGRYPAPG 250
251 QDPSMVQTYYPGALKAVRDAEAALNVPSNKKLHVQFMSSKWDSGDPRSNA 300
301 AVKNDPMVGFDDHNYIGFALSNTGDQYSLMHSACTDSRVVSGQDFAITGE 350
351 WSMTSGADWHDGNFFTKFFTAQQQLYESPGMDGWIYWTWKTELNDPRWTY 400
401 SYATYLNYIPTNAAALQQQNVYQDVCSGYR                     430
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Fig. 1. Amino acid sequence of *T. harzianum* pustulanase (ThBGL1.6). Signal peptides, predicted by the SignalP tool (www.cbs.dtu.dk/services/SignalP/), is shown in small letters; matching tryptic peptides identified by MALDI-TOF MS are shown in bold; the potential N-glycosylation sites are shown in boxes. Conserved putative catalytic structure is underlined.

Table 1
Specific activities (U/mg protein) of crude enzyme preparations toward different substrates (50 °C, pH 5.0).

Enzyme preparation	Pustulan	MCC	β-glucan	pNPG[a]
PV-control	0.2 ± 0.01	0.6 ± 0.05	15 ± 1.5	1.1 ± 0.1
PV4	46 ± 4	0.4 ± 0.04	8 ± 0.8	0.8 ± 0.07
PV15	80 ± 7	0.3 ± 0.03	6 ± 0.5	0.9 ± 0.09
PV35	66 ± 6	0.5 ± 0.05	10 ± 1	1.1 ± 0.1
PV44	71 ± 7	0.3 ± 0.03	8 ± 0.8	1.7 ± 0.1

[a] Activity was measured at 40 °C and pH 5.0.

protein components and salts, was fractionated by AEC on a Source 15Q column at pH 6.8 (Fig. S3, Supplementary data). The elution profile contained a non-bound protein fraction and 4 major peaks observed at the beginning of 1 M NaCl gradient. The third and fourth major fractions eluted at 0–10% NaCl, marked with an arrow in Fig. S2, displayed the highest activity against pustulan. However, according to the SDS-PAGE data, this fraction contained several protein bands (data not shown). So, it was further separated using HIC on a Source 15 Isopropyl column (Fig. S4) followed by gel-filtration on a Superdex-75 column (data not shown). As a result, a homogeneous ThBGL1.6, according to the data of SDS-PAGE and IEF, was obtained (Fig. 2).

The ThBGL1.6 had an apparent molecular mass of 45 kDa and pI5.6. The molecular mass of the pustulanase observed in the SDS-PAGE is approximately the same as the mass theoretically calculated which is proved by the absence of possible protein N-glycosylation.

3.4. Substrate specificity and kinetic parameters of purified ThBGL1.6

Specific activities of purified ThBGL1.6towards different substrates are shown in Table 2. The enzyme displayed high activity toward pustulan – 116 U/mg of protein. The ThBGL1.6 also displayed low activity toward CMC and barley β-glucan (3.6 and 4.3U/mg of protein, respectively), while the specific activity against laminarine and gentiobiose was not detected. The obtained data demonstrate the high specificity of the enzyme against substrates containing the (1,6)-β-glucosidic linkages. However, it should be noted that the enzyme is not active against Ge, which is probably due to endo-depolymerase type of ThBGL1.6 action.

K_m and k_{cat} for ThBGL1.6weredeterminedin hydrolysis of pustulan. K_m and k_{cat} were 1.0 g/L and 30 s^{-1}, respectively (pH 5.0, 50 °C). The observed values of kinetic parameters were comparable to those reported for GH5 family enzymes from other microorganisms. For example, the K_m values for enzymes from *T. harzianum BGN16.1, Acremonium persicinum* and *P. brefeldianum* were 0.8, 1.28 and 2.78 g/L, respectively [5,31,32].

3.5. Temperature and pH-dependences of activity and thermostability

Effect of pH on the activity of ThBGL1.6 is shown in Fig. 3A. The maximum activity of ThBGL1.6 was observed at pH 5.0; more than 80%

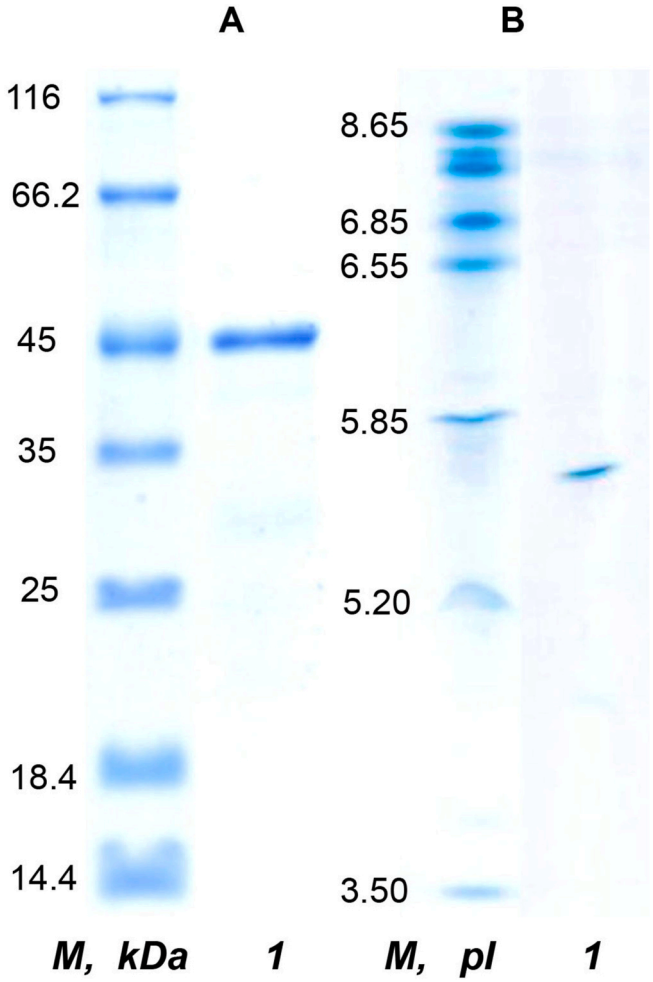

Fig. 2. SDS-PAGE (A) and IEF (B) of purified BGLs. *1*, ThBGL1.6.

of the activity was retained at pH 4.60–5.85. The ThBGL1.6 displayed bell-shaped form of the enzyme activity dependence on pH. The observed pH-optima for the enzyme was similar to those reported for other fungal pustulanases [5,33]. The temperature optimum for ThBGL1.6 was 50 °C observed at pH 5.0, as shown in Fig. 3B.

Thermostability studies showed that the ThBGL1.6 was stable at 40–45 °C and pH 5.0: no decrease in activity was observed during at least 3 h of incubation (Fig. 3C); while at 50 °C the enzyme retained 60% of the activity after 3 h of incubation. At 55 and 60 °C, the half-life time of ThBGL 1.6 was 10 min and 2 min, respectively.

Table 2
Specific activities (U/mg protein) of pure ThBGL1.6 against different substrates (50 °C, pH5.0).

Substrate	Linkage type	ThBGL1.6
Pustulan	β-1,6	166 ± 15
CMC	β-1,4	3.6 ± 0.4
β-glucan	β-1,4	4.3 ± 0.4
Xylan	β-1,4	0
Colloidal chitin	β-1,4 (GlcNAc)	0
Dextran	α-1,3	0
Soluble starch	α-1,4: α-1,6	0
Laminarin	β(1 → 3):β(1 → 6)	0
Gentiobiose (Ge)[a]	β-1,6	0
Curdlan	β-1,3	0

[a] Activity was measured at 40 °C.

3.6. The mechanism of pustulan hydrolysis by recombinant ThBGL1.6

The prolonged pustulan hydrolysis by the homogeneous *ThBGL1.6* was carried out during 48 h at 40 °C in Na-acetate buffer, pH 5.0. The depth of hydrolysis of pustulan during incubation with enzyme was almost 40% based on the RS. A minimum pustulanase dose (0.1 U/mL) was used to detect changes in the molecular weight distribution (MWD) of the substrate in the pustulan hydrolysis course. In Fig. S5, the retention time (r.t., [min]) of high-molecular peak of the pustulan was 12.5 r.t. (with trace amounts of glucose). After 5 min of pustulan hydrolysis it was detected a mixture of oligosaccharides (DP > more than 5) with a 15 min r.t., a small concentration of gentiobiose (18 min r.t.) and glucose (18.5 min r.t.). After 30 min, the hydrolysis products and character of MWD changed. The height of the high-molecular peak of pustulan twice decreased (12.5 min r.t.), while a mixture of oligosaccharides (DP 2–4) eluting after 16.5 min r.t. was appeared. After 3 h of hydrolysis, the peak elution of pustulan and oligomeric fractions with DP > 3 almost disappears, while the yield of gentiotriose almost twice increased (17.5 min r.t.) and the concentration of Ge increased more than 4 times (18 min r.t.). After 24 h of hydrolysis, the qualitative set of pustulan destruction products practically didn't change. Therefore, we can conclude that the main products of pustulan hydrolysis with ThBGL1.6 are Ge and gentiotriose in the ratio of approximately 2:1 with trace amounts of oligosaccharides DP 4–7 and glucose. The obtained experimental results confirmed the endo-depolymerase activity of ThBGL1.6 against pustulan.

3.7. Progress kinetics of pustulan

Pustulan (5 g/L) was hydrolyzed by homogenous ThBGL1.6 at a concentration in the reaction mixture 0.1, 0.5 and 1.0 U/mL. Samples were taken at certain intervals from start of the process, the RS concentration was determined, and the composition of the hydrolysis products was also evaluated by HPLC-PAD. Progress kinetics of pustulan hydrolysis by the purified ThBGL1.6 was studied (Fig. 4).

On the first stage of the reaction, the intensive RS formation was observed. After 30 min of the reaction the concentration of RS in the reaction mixture for enzyme dosage 0.1, 0.5 and 1.0 U/mL was 0.9, 1.85 and 2.15 g/l, respectively. According to the HPLC data the major hydrolysis product was gentiobiose (Ge, 70–80% of the total content of the RS), the other products were oligosaccharides with a higher degree of polymerization (DP) – mainly with DP 3 and 4; oligosaccharides with DP5-40 were observed only in the samples taken 5 min after initiation of the hydrolysis process). At a dosage of ThBGL1.6 0.1 U/ml of the enzyme, the hydrolysis process was almost completed in 3 h, with higher doses, a significant slowdown of the process was observed after 3 h. After 24 h of the reaction the RS yield for the enzyme doses of 0.1, 0.5 and 1.0 U/ml was 2.0, 2.5 and 2.8 g/L (the degree of substrate conversion was 35–50%). The composition of the hydrolysis products was as follows: 94–99% of Ge and 1–6% of gentio-oligosaccharides (mainly G3 and a small amount of G4-G8, which confirms the activity of ThBGL1.6 against oligosaccharides with DP > 4, Fig. S6). The structure of pustulan and the main reaction product of hydrolysis (gentiobiose, Ge) is shown in Fig. S7.

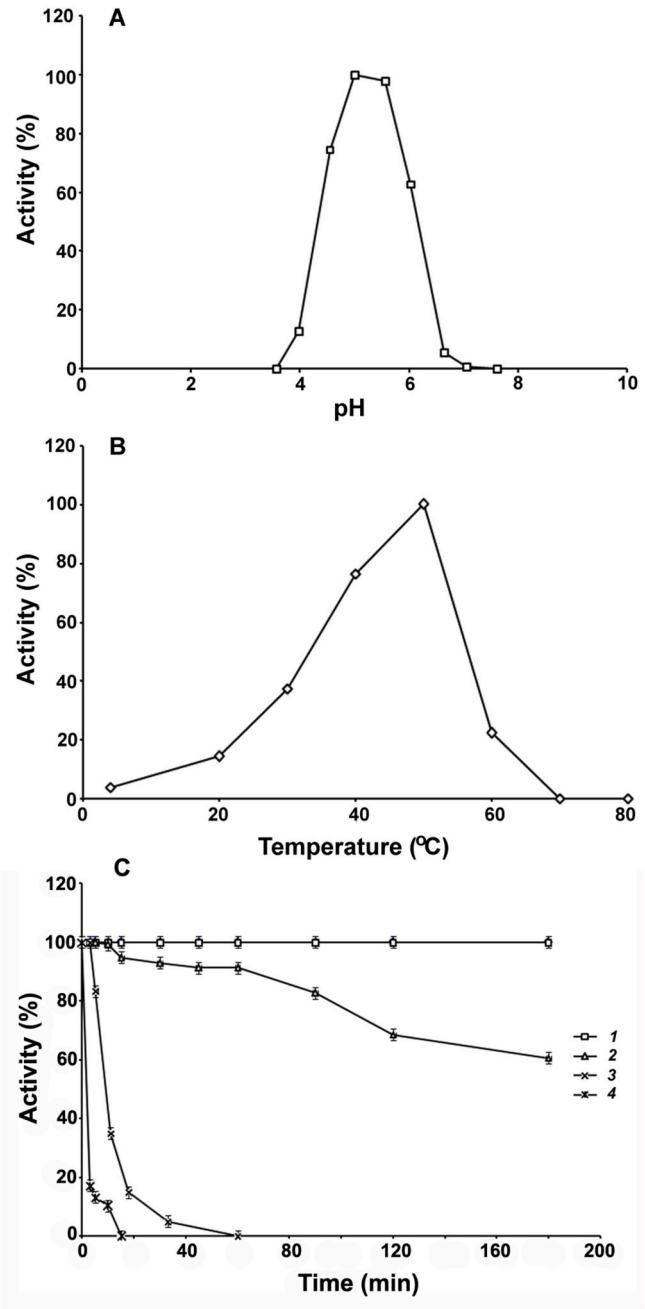

Fig. 3. Effect of pH (A), temperature (B) on ThBGL1.6 activity and its thermostability (C) at pH 5.0 (0.1 M Na-acetate buffer). Enzyme was incubated at 45 (1), 50 (2), 55 (3) and 60 (4) °C. Sample aliquots were taken at definite times of incubation at certain temperature, in which a residual pustulanase activity was determined under standard conditions.

As already shown, the degree of pustulan hydrolysis did not exceed 35–50%. As far as we studied the pustulanase ThBGL1.6 did not activate against Ge and G3, it was suggested that adding an exo-glycosidase with activity against β-1,6-glycosidic bond to the reaction mixture at the initial stage of the process would improve the process of pustulan conversion. For this purpose, we used β-glucosidase of *A. niger* (rAnBGL): in our early study we showed that rAnBGL was active against Ge (147 U/mg of protein) [34].

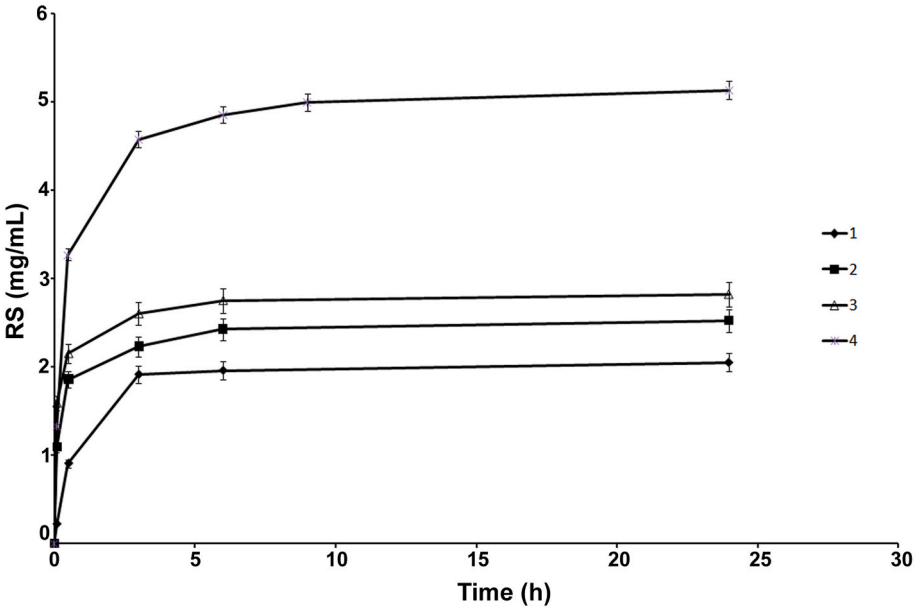

Fig. 4. Progress kinetics curves of pustulan (5 g/L) hydrolysis by purified ThBGL1.6 (*1*, 0.1 U/mL; *2*, 0.5 U/mL; *3*, 1 U/mL and *4*, 0.5 U/mL + 0.5U/mL of rAnBGL) at 40 °C.

The addition of rAnBGL to the reaction mixture remarkably increased the total RS yield and initial velocity of pustulan conversion process (Fig. 4) up to 100% the pustulan conversion after 24 h (when 0.5 U/mL rAnBGL was used, Fig. 4).

In the case of simultaneous use of rAnBGL (0.5 U/mL) and ThBGL1.6 (0.5 U/mL) after 30 min of the reaction the RS yield was 3.27 g/L, which is equal to the 59% of the pustulan conversion and which is almost 1.6 times higher than in the case of ThBGL1.6 individual use. The yield of glucose, Ge and oligosaccharides (DP3-7) was 1.54, 1.16 and 0.57 g/L, respectively. After 3 h the RS yield was 4.57 g/L (82% of pustulan conversion). Glucose yield increased significantly (2.66 g/L), whereas the Ge yield decreased to 0.18 g/L. The RS concentration after 24 h was 5.13 g/L, which is comparable with 92% of pustulan conversion. Glucose (3.5 g/l) in this case is the main component of RS, and only trace amounts of Ge (0.1 g/l) and oligosaccharides with DP 3–5 were detected.

4. Conclusions

A novel recombinant ThBGL1.6 was purified in a homogeneous form. The enzyme exhibited a high activity against pustulan by the endo-depolymerase type of action. It also demonstrated a minor activity to CMC and β-1,3/1,4-glucan from barley, but ThBGL1.6 was not active against Ge and gentiotriose. At studying of a long-term pustulan hydrolysis by purified ThBGL1.6, it was found that the main reaction product was Ge. It should be underlined that Ge is a valuable product that can be applied as an inductor of transcription of the cellulase pool of fungal enzymes [35], also it can be used as a substrate to determine the activity of some enzymes, such as β-glucosidase.

On the other hand, to achieve the most complete pustulan conversion to glucose along with pustulanase (ThBGL1.6) in the hydrolysis mixture it is necessary to add β-glucosidase. This approach will allow convert all hydrolysis products into glucose, which can serve as a source of biotechnological products with added value.

CRediT authorship contribution statement

P.V. designed plasmids, expressed and characterized the recombinant GH5 family endo-β-1,6-glucosidase (ThBGL1.6) from *T. harzianum* and was responsible for the concept of the study. **A.M.** planned of the experiments. **O.S., KF** and **I.Z.** purified homogeneous proteins and edited a foreign language of manuscript. **A.S.** discussed the results and revised manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The work was supported by the Ministry of Science and Higher Education of the Russian Federation (State task #0104-2019-0009).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2020.108211.

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