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Enzymological Properties of Endo-(1–4)-β-Glucanase Eg12p of *Penicillium canescens* and Characteristics of Structural Gene *egl2*

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Abstract—Gene *egl2* of secreted endo-(1–4)-β-glucanase of glycosyl hydrolase family 5 of the mycelial fungus *Penicillium canescens* was cloned. The gene was expressed in *P. canescens* under control of a strong promoter of the *bgaS* gene encoding β-galactosidase of *P. canescens*, and endoglucanase producing strains were obtained. Chromatographically purified recombinant 48 kDa protein had pH and temperature optima 3.4 and 60°C, respectively, exhibited specific activity of 33 IU, and had K_m and V_{max} in CM-cellulose hydrolysis of 10.28 g/liter and 0.26 μmol/sec per mg, respectively.

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Key words: endoglucanase, mycelial fungus, *Penicillium canescens*, gene

Cellulose is the most widespread polysaccharide in the biosphere, which accounts for its wide use as a raw material for processing and production of energy and a broad spectrum of substances. The primary treatment of cellulose includes chemical or enzymic hydrolysis, and the latter is preferable because it makes it possible to carry out the process at a lower temperature and to perform directed synthesis of target products. The enzymic hydrolysis of cellulose is catalyzed by a complex of enzymes of four types: endo-(1–4)-β-glucanase (EC 3.2.1.4), exo-(1–4)-β-glucanase (EC 3.2.1.91), exo-(1–4)-β-glucosidase (EC 3.2.1.74), and cellobiase (EC 3.2.1.21).

Endo-(1–4)-β-glucanase hydrolyzes internal 1–4-β-glycoside bonds remote from the ends of the cellulose polymer chain with formation of cellooligosaccharides. The effects of endoglucanase are characterized by sharp decrease in the extent of polysaccharide substrate polymerization [1].

As a rule, endoglucanases are monomeric globular proteins. Molecular mass values of fungal endoglucanases are in the range 11-100 kDa, but the most typical are 30-55 kDa. Molecular mass values of bacterial endoglucanases are usually somewhat higher and exceed 65 kDa [2]. Most described fungal endoglucanases are acidic proteins (p*I* 2.7-5.0).

Endoglucanases are applied in different fields of industry such as production of bioethanol, textile, food, and fodder industry, processing of secondary raw materials, and production of biological fuel. This defines the practical significance of investigations aimed at design of new endoglucanase-producing strains.

Characteristics of described endoglucanases not always meet requirements for used technological regimes, and therefore it is doubtless that obtaining endoglucanases with new physicochemical properties is an important endeavor.

The aim of this work was to isolate the full-sized gene of the *Penicillium canescens* endo-(1–4)-β-glucanase of glycosyl hydrolase family 5, obtaining recombinant protein and its biochemical characterization for evaluation of possibilities of its future practical application.

MATERIALS AND METHODS

Strains of microorganisms. The *P. canescens* strain F178 (All-Russian Collection of Industrial Microorgan-

Abbreviations: CMC, CM-cellulose; DSC, differential scanning calorimetry.

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isms) and its PCA-10 mutant at the nitrate reductase gene (*niaD*$^-$) [3] were used. The *Escherichia coli* strain XL-1 Blue (Stratagene, USA) was used to construct plasmids.

The technique of protoplast formation and PCA-10 strain co-transformation as well as methods of work with recombinant DNA are described elsewhere [4].

Culture growth. To obtain spore inoculation material, culture of *P. canescens* strains was grown on agarized Rowlen–Toma medium at 30°C for 5-7 days. This medium with sucrose has the following composition (g/liter): sucrose, 20; $NaNO_3$, 6; KH_2PO_4, 2; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; agar, 20; mixture of microelements, 1 ml; distilled water, to 1 liter. Mixture of microelements consists of (mg/liter): $MnSO_4 \cdot 5H_2O$, 800; $CuSO_4 \cdot 5H_2O$, 400; $ZnSO_4 \cdot 7H_2O$, 800; $FeSO_4 \cdot 7H_2O$, 800; H_3BO_3, 50; $(NH_4)_2MoO \cdot 4H_2O$, 800. Aqueous suspension of conidia ($4 \cdot 10^5$ conidia/ml) was inoculated into 100 ml fermentation medium of the following composition (g/liter): beet pulp, 30; peptone, 50; KH_2PO_4, 25; distilled water, to 1 liter, pH 4.5.

Growth was continued in flasks on an orbital shaker at 240-250 rpm for 120 h at 30°C. When fermentation was over, the culture liquid was separated from mycelium and solid fragments by centrifuging (10,000*g*, 15 min, 20°C), then endoglucanase and β-galactosidase activities in the supernatant were determined.

Purification of endoglucanase. Purification of *P. canescens* endoglucanase (Egl2p) of glycosyl hydrolase family 5 included three steps: preliminary purification, anion-exchange, and hydrophobic chromatography on an FPLC liquid chromatograph (Pharmacia, Sweden).

The preliminary step of purification included pigment and salt removal from culture liquid of the mycelial fungus *P. canescens* by gel filtration on a 5 × 50 cm column with carrier Bio-Gel P2 (Bio-Rad, USA) using 10 mM Bis-Tris-HCl, pH 7.0, as the eluent.

Anion-exchange chromatography was carried out on a 2.5 × 8 cm column with carrier Source 15Q (GE-Healthcare, Sweden) equilibrated with 10 mM Bis-Tris-HCl, pH 7.0. Carrier-bound proteins were eluted with a 0-1 M NaCl linear gradient (2% per column volume).

Hydrophobic chromatography was carried out on a 1.6 × 10 cm column with carrier Source 15ISO (GE-Healthcare) equilibrated with 1.7 M $(NH_4)_2SO_4$ solution in 50 mM Na-acetate buffer, pH 5.0. Carrier-bound protein was eluted in a linear 1.7-0 M $(NH_4)_2SO_4$ gradient (4% per column volume).

After desalting the fractions on 0.8 × 3 cm columns with Bio-Gel P2, the proteins were analyzed by SDS-PAGE and endoglucanase activity was determined.

Determination of enzyme activities. Endoglucanase activity was determined by cleavage of soluble CM-cellulose stained with "active orange" (AO-CMC). The stained substrate solution (1%) in 0.1 M acetate buffer, pH 5.0, was incubated with the enzyme for 10 min at 40°C. After incubation, the substrate was precipitated with 1 M $CaCl_2$ solution in 80% alcohol and centrifuged at 5000*g* for 5 min at 20°C. The dye concentration in solution was registered on a spectrophotometer at 490 nm. The resulting values were converted into international units (IU) after determination of the coefficient of correlation with CM-cellulase activity. The enzyme amount that forms 1 μmol of reducing sugars in 1 min under the given reaction conditions was taken as the activity unit.

For determination of β-galactosidase activity, reaction mixture containing 3 mg *o*-nitrophenyl-β-galactopyranoside (*o*NPG) in 1.9 ml 0.05 M Na-acetate buffer, pH 4.5, and 100 μl culture liquid of corresponding dilution was incubated for 15 min at 30°C, then 1 ml 1 M Na_2CO_3 was added and optical density at 420 nm was determined. The β-galactosidase amount that releases from *o*NPG substrate 1 μmol of *o*-nitrophenol in 1 min under the given reaction conditions was taken as the activity unit [5].

Determination of protein concentration. Protein concentration was determined using the BSA Protein Assay kit (Pierce, USA) with BSA as standard.

Determination of enzyme molecular mass and homogeneity. The molecular mass and homogeneity of the enzyme was determined by electrophoresis under denaturing conditions according to Laemmli [6]. Protein electrophoresis was carried out in denaturing 12% SDS-polyacrylamide gel on a Mini Protean III device (Bio-Rad). Protein bands were stained with Coomassie Brilliant Blue R-250. The protein mixture Page Ruler™ Prestained Protein Ladder (Fermentas, Lithuania) in the range of 10-200 kDa was used as the standard.

Determination of temperature and pH optima for Eg12p activity and thermal stability. The pH profile of enzyme activity was determined by measuring enzyme activity in the pH range from 3 to 6. A universal buffer (mixture of phosphoric, acetic, and boric acids, 0.1 M each, pH adjusted to required value with 0.2 M NaOH) was used to prepare solutions with the assigned pH values.

Temperature profile of enzyme activity was determined by measuring enzyme activity at different temperatures in 0.1 M sodium acetate buffer, pH 4.5.

For determination of thermal stability, the time dependence of endoglucanase activity towards the AO-CMC substrate (Fermentas) at 50°C was studied.

Results of measurements are expressed as percent of maximal activity taken as 100%.

Determination of kinetic parameters. Values V_{max} and K_m were calculated from experimental concentration dependences of initial rate of CM-cellulose hydrolysis. The Somogyi–Nelson technique was used for determination of the extent of hydrolysis by accumulation of reducing sugars [7, 8]. The enzyme concentration in the sample was 0.29 μg/liter, pH 4.5, 40°C. The results were processed in Lineweaver–Burk double reciprocal coordi-

nates [9]. Since exact molar concentration of polymeric substrates cannot be determined, Michaelis constant K_m is expressed in g/liter.

Differential scanning calorimetry. Calorimetric behavior was studied using a DASM-4M differential scanning adiabatic microcalorimeter (Special Design Bureau, Russian Academy of Sciences) with 430 μl capillary platinum cells. To obtain heat absorption curves, recombinant Egl2p in 50 mM K-phosphate buffer, pH 5.5, was placed into the cell and heated at the rate of 1°C/min from 20 to 76.8°C under constant pressure of 2 atm. The reference cell contained the same buffer solution. Calibration capacity was 25 μW.

RESULTS AND DISCUSSION

Cloning the *egl2* gene of *P. canescens* 1–4-β-endoglucanase of glycosyl hydrolase family 5. To clone an internal fragment of the *P. canescens* gene *egl2*, primers to conserved sites of glycosyl hydrolase family 5 endo-(1–4)-β-glucanase genes were used: eg2dir1, 5′-ATCATGAACGAGCCCCATGA-3′; eg2dir2, 5′-GACAATGTGGTGGTCAGGGATG-3′; eg2dir3, 5′-CTCGAGGTTTGACTAACGCTGA-3′; eg2rev1, 5′-CTGTTGTCACTATCCAGGTACTTGTG-3′; eg2rev2, 5′-AGTTCTTCCACTTGGGGACG-3′. Different combinations of forward and reverse primers were used for the *P. canescens egl2* gene amplification by PCR, but a positive result was obtained only for the pair of primers eg2dir2 and eg2rev1.

An amplified DNA fragment of about 900 bp was cloned in the plasmid vector pUC19 cleaved by the *Sma*I restriction site and sequenced. Base sequence analysis of the *P. canescens egl2* gene fragment revealed a significant homology between this fragment and genes of the glycosyl hydrolase family 5 endoglucanases of mycelial fungi.

The 5′ and 3′ flanking sequences of the fragment were cloned using the "PCR of uncloned genomic DNA" technique [10] with primers specific of the detected sequence of the internal gene fragment: EG2-5′-1, 5′-CGTAGAGGTAGTTGGAGCAACAGTTGT-3′; EG2-5′-2, 5′-AATTATCAATTGTCAACGAAGGGTACA-3′; EG2-3′-1, 5′-TGGACTAGCGCTGGAAACTTTATCGAA-3′; EG2-3′-2, 5′-GATTTTGCTCCCCGGTACAGACTGGACTA-3′. This resulted in amplification and partial sequencing of extended 5′ and 3′ non-translated regions of the *P. canescens egl2* gene of about 2.5 and 3 kb, respectively (Fig. 1). Nucleotide and amino acid sequences of *P. canescens egl2* gene are shown in Fig. 2.

Gene structure. The coding region of the endoglucanase gene is divided by a single intron whose position was detected by comparison with genes of other endoglucanases of this family and using conserved *P. canescens* sequences at the intron beginnings and ends in mycelial fungi [11]. The signal peptide of *P. canescens* endo-

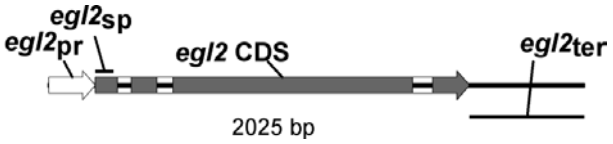

Fig. 1. Map of *P. canescens* gene *egl2*. Promoter (pr), signal peptide encoding sequence (sp), coding region (CDS), and gene terminator (ter) are designated.

(1–4)-β-glucanase consists of 21 amino acids, and the site of signal peptide detachment was determined by comparison of amino acid sequences of the glycosyl hydrolase family 5 fungal endoglucanases (Fig. 3) and by computer modeling. The base sequence translation showed that the mature protein consisting of 388 amino acids and has molecular mass 41.5 kDa and p*I* 5.28.

Expression of the endoglucanase gene. The strong promoter of the *bgaS* gene of the *P. canescens* β-galactosidase and leader peptide of the latter were used for expression of gene *egl2* in the fungus [5].

The fragment of the *P. canescens* gene *egl2* containing the encoding sequence (without signal peptide) and its own terminator was amplified using *Pfu*I polymerase and a pair of primers with inserted restriction sites: EG2ApaI, 5′-CGC**GGGCCC**AGCAGTCGGCTTGGGGACAAT-3′; EG2NotI, 5′-AAA**GCGGCCGC**TGTGTGATATCGGCATGTGCG-3′; a preparation of chromosomal DNA of *P. canescens* was used as the template. The fragment synthesized by PCR was treated with restriction endonucleases *Apa*I and *Not*I and was then cloned in plasmid pPCBG5 containing the complete *bgaS* gene instead of the initial coding region and terminator with preservation of reading frame (Fig. 4). The inserted fragment was checked by sequencing for absence of mutations.

The resulting plasmid pBGEG2 carrying endoglucanase gene *egl2* under control of the gene *bgaS* promoter with its leader peptide, was introduced into the genome of the *P. canescens* PCA-10 strain mutant at the nitrate reductase gene (*niaD*) by means of co-transformation [3] with plasmid pSTA-10 [12] carrying the complementary gene *niaD* of *Aspergillus niger*.

Twenty transformant clones were selected on selective medium containing $NaNO_3$ as a source of nitrogen. Nine of these transformants exhibited increased endoglucanase activity after growth. In the most productive strains, endoglucanase activity exceeded 30-40-fold that in the recipient strain PCA-10 (table). Figure 5 shows the SDS-PAGE pattern of proteins of culture liquids of the recipient strain PCA-10 and recombinant strain EGL2-4. The appearance of a 48 kDa protein product and lowered intensity of the band corresponding to β-galactosidase are well seen in the recombinant strain proteins, which correlated with measurements of the enzyme activity. The

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   1  AGCGTCTTCG TCAGGGGCTG CTTCCGTGTC GTAGAGAGGC AAATTAGATC TGAACATGAG TATTACCCCA CTGTATCAAG

  81  GGATTGTTGT TTGATAATTC AGGAGAAGAT ATAAAGACCC CCGCTATGTC CCTTTGCGAT ACCTGAAACC AGTCTATCGG

                      M  R  D  Q  H  K  Q  S  L  F  A  L  L  A  I  S  G  S  A  L  G  Q
 161  AATACTACCT TCAAAATGAG AGACCAGCAC AAGCAGAGCC TGTTCGCACT CCTTGCCATT AGCGGATCAG CGCTAGGGCA

        Q  S  A  W  G  Q  >================= intron 1 ============================<C  G  G
 241  GCAGTCGGCT TGGGGACAAT GTTGGTTATA TAATCCAGAT ATATGAGCGA GAGGCTAATG AGGATGATTT AGGTGGTGGA

       Q  G  W  T  G  Q  T  T  C  V  S  G  Y  Y  C  S  A  Q  N  A  W  Y  S  Q  C  I  P
 321  CAGGGCTGGA CTGGCCAGAC GACCTGCGTG TCTGGATACT ACTGCTCTGC CCAAAACGCT TGGTATTCGC AGTGCATTCC

        G  S  >========================= intron 2 ============================<G  G  G  S
 401  AGGCTCGGGT GAGTGACCCA ACATATACCT GGAATTGTAC CCTTCGTTGA CAATTGATAA TTGAGTAGGC GGTGGTAGCG

       G  T  T  T  T  L  K  T  T  T  T  V  A  P  T  T  S  T  T  T  S  S  S  S  V  P
 481  GTACTACCAC TACCCTGAAA ACCACTACAA CTGTTGCTCC AACTACCTCT ACGACGACCT CTTCGAGCTC TTCCGTGCCA

       T  G  K  V  R  F  A  G  V  N  I  A  G  F  D  F  G  M  V  T  S  G  T  Q  D  Q  S
 561  ACGGGTAAAG TCCGATTTGC GGGTGTCAAC ATCGCTGGCT TCGACTTTGG TATGGTGACC AGTGGTACTC AGGACCAGAG

        Q  V  Y  D  I  S  G  D  G  V  N  Q  M  R  H  F  V  N  D  D  T  F  N  M  F  R
 641  CCAGGTCTAT GACATATCAG GCGACGGTGT CAATCAAATG AGGCACTTTG TCAACGATGA TACCTTCAAC ATGTTCCGCC

       L  P  T  G  W  Q  F  L  V  A  N  N  L  G  G  S  L  D  S  N  N  F  G  K  Y  D  K
 721  TGCCGACAGG ATGGCAGTTC CTCGTTGCTA ATAACCTGGG TGGCTCTCTG GACTCGAACA ACTTCGGCAA GTATGACAAG

       L  V  Q  G  C  L  S  L  G  A  Y  C  I  I  D  I  H  N  Y  A  R  W  N  G  A  V  I
 801  TTGGTTCAAG GCTGTCTGTC CCTGGGCGCT TATTGCATCA TTGATATCCA CAACTATGCC CGTTGGAACG GTGCCGTCAT

        G  Q  G  G  P  T  D  A  Q  F  V  A  L  W  T  S  L  A  T  K  Y  K  S  Q  S  K
 881  TGGACAAGGC GGTCCAACAG ATGCGCAATT CGTCGCCCTT TGGACATCAC TTGCGACCAA ATACAAGAGC CAGAGCAAAA

       I  V  F  G  V  M  N  E  P  H  D  V  D  I  K  T  W  A  A  T  V  Q  K  V  V  T  A
 961  TCGTATTCGG CGTTATGAAT GAGCCTCATG ACGTCGATAT CAAAACATGG GCTGCTACGG TACAGAAGGT CGTGACCGCG

       I  R  N  A  G  A  T  S  Q  M  I  L  L  P  G  T  D  W  T  S  A  G  N  F  I  E  N
1041  ATCAGAAATG CTGGGGCCAC GTCTCAAATG ATTTTGCTCC CCGGTACAGA CTGGACTAGC GCTGGAAACT TTATCGAAAA

        G  S  G  A  A  L  S  A  V  V  N  P  D  G  S  T  T  N  L  I  F  D  V  H  K  Y
1121  TGGCTCTGGC GCCGCGCTCT CAGCGGTGGT CAACCCAGAT GGATCTACTA CTAATTTGAT ATTTGATGTG CACAAGTACC

       L  D  S  D  N  S  G  T  H  A  E  C  T  T  N  N  V  D  V  F  N  N  L  G  Q  W  L
1201  TCGACAGTGA CAACAGTGGC ACCCACGCCG AGTGCACGAC CAACAATGTC GACGTATTTA ACAATCTCGG ACAATGGCTA

       R  S  N  K  R  Q  A  I  L  S  E  T  G  G  G  N  V  Q  S  C  A  T  N  M  C  Q  Q
1281  CGCTCAAACA AGCGGCAAGC TATTCTTAGC GAGACTGGCG GCGGTAATGT CCAAAGCTGT GCAACGAACA TGTGTCAGCA

        L  D  A  L  N>================================== intron 3 ======================
1361  GCTGGATGCC CTCAAGTAAG TACCATCTTG GCCCTATCCA AGTGATCGAT TAAGCGGAAT CCCGTGGAAC ATCTATACTG

      ===========<  A  N  S  D  V  Y  L  G  W  T  S  W  S  A  G  A  F  L  T  S  Y  V  L
1441  ACTTCACCCA GTGCAAACTC GGATGTTTAT CTCGGTTGGA CCTCTTGGAG TGCTGGTGCC TTCCTGACAT CCTATGTTCT

        S  E  V  P  T  N  N  V  D  Q  Y  L  V  Q  Q  C  F  V  P  K  W  K  N  &
1521  GTCTGAAGTG CCGACCAATA ACGTTGATCA GTACCTCGTT CAACAGTGCT TTGTCCCTAA GTGGAAGAAC TGAGTGCTAG

1601  GTGTTTGTTA CTGCATCCCG TCTCTTTTTC TAAAGAAGGA ATGACCGATC AGTGCCGTTG AAACAGGATC AGATGGACTT

1681  AGATTACAAG GTATCAAAAT ACAGCGTCCC TTTCTCTGGA ATTTAGCCTT ATTTCCATTT GAGCGGAAAA TGTACTCTGG

1761  GATTCCGCCT AATTATAAAT GCTTGTTCCC AATGGTAGCT GTTGACATAG AATGAAGATA CAGGAGACTT ACCATCCCCA

1841  ACTGCTATCG ACTTCTAGCT TCATTAGAGT GTTCCAATGG GAGATATTGA TATTATCTAC AATACAATTG ATCAACACGG

1921  AGATTGACAC TTATTTAGAC CCCCGTTAGA TGTCTCCTCA CGTGACGTTT TGTATGCATG ACACGTGACC TATGACATAA

2001  GTCGCACATG CCGATATCAC ACACC
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Fig. 2. Nucleotide and amino acid sequences of *P. canescens* endo-(1–4)-β-glucanase gene *egl2*. The signal peptide sequence is underlined.

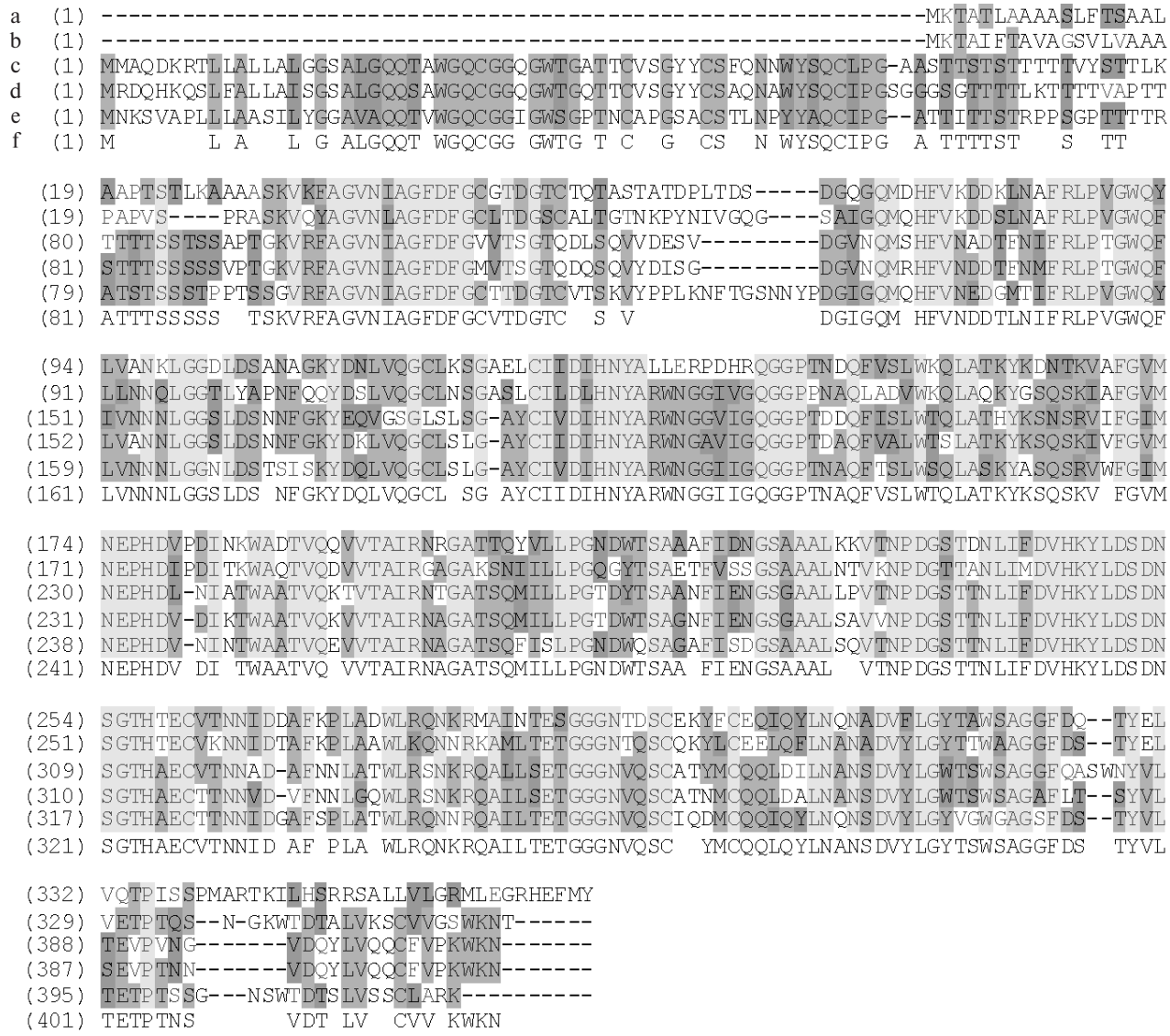

Fig. 3. Comparison of amino acid sequences of the glycosyl hydrolase family 5 endo-(1–4)-β-glucanases of mycelial fungi: a) *Macrophomina phaseolina*; b) *Phaeosphaeria nodorum*; c) *Penicillium janthinellum*; d) *Penicillium canescens*; e) *Trichoderma reesei*; f) consensus sequence.

decreased amount of β-galactosidase can be explained by titration of the gene trans-activator due to increased promoter copy number. The difference of the recombinant protein molecular mass from the theoretically calculated value 43.5 kDa suggests a significant extent of glycosylation of the enzyme.

Isolation of homogenous Eg12p from *P. canescens* culture liquid. After purification from non-protein contaminants by gel filtration on Bio-Gel P2, the enzyme preparation was purified by anion-exchange chromatography on a Mono Q column. Endoglucanase activity was detected in fractions not bound to the carrier. According to data of SDS-PAGE, these fractions contained about 20% contaminating proteins.

The enzyme was further purified by hydrophobic chromatography on a Source 15ISO column. Endoglucanase activity was detected in fractions eluted in $(NH_4)_2SO_4$ gradient in the range of 1.35-1.45 M. The homogeneity of the resulting Eg12p was confirmed by SDS-PAGE (Fig. 5).

The purification procedure yielded a homogeneous Eg12p preparation with specific activity of 33 IU. Protein yield was 2% with 16.6-fold increased specific activity.

According to SDS-PAGE, the molecular mass of homogeneous Eg12p is 48 ± 1 kDa. Comparative analysis of molecular mass values of recombinant endoglucanases of glycosyl hydrolase family 5 showed that our preparation of recombinant Eg12p has a relatively high molecular mass compared to those described in the literature [13, 14].

Determination of physicochemical parameters of Eg12p. Endoglucanases of the glycosyl hydrolase family

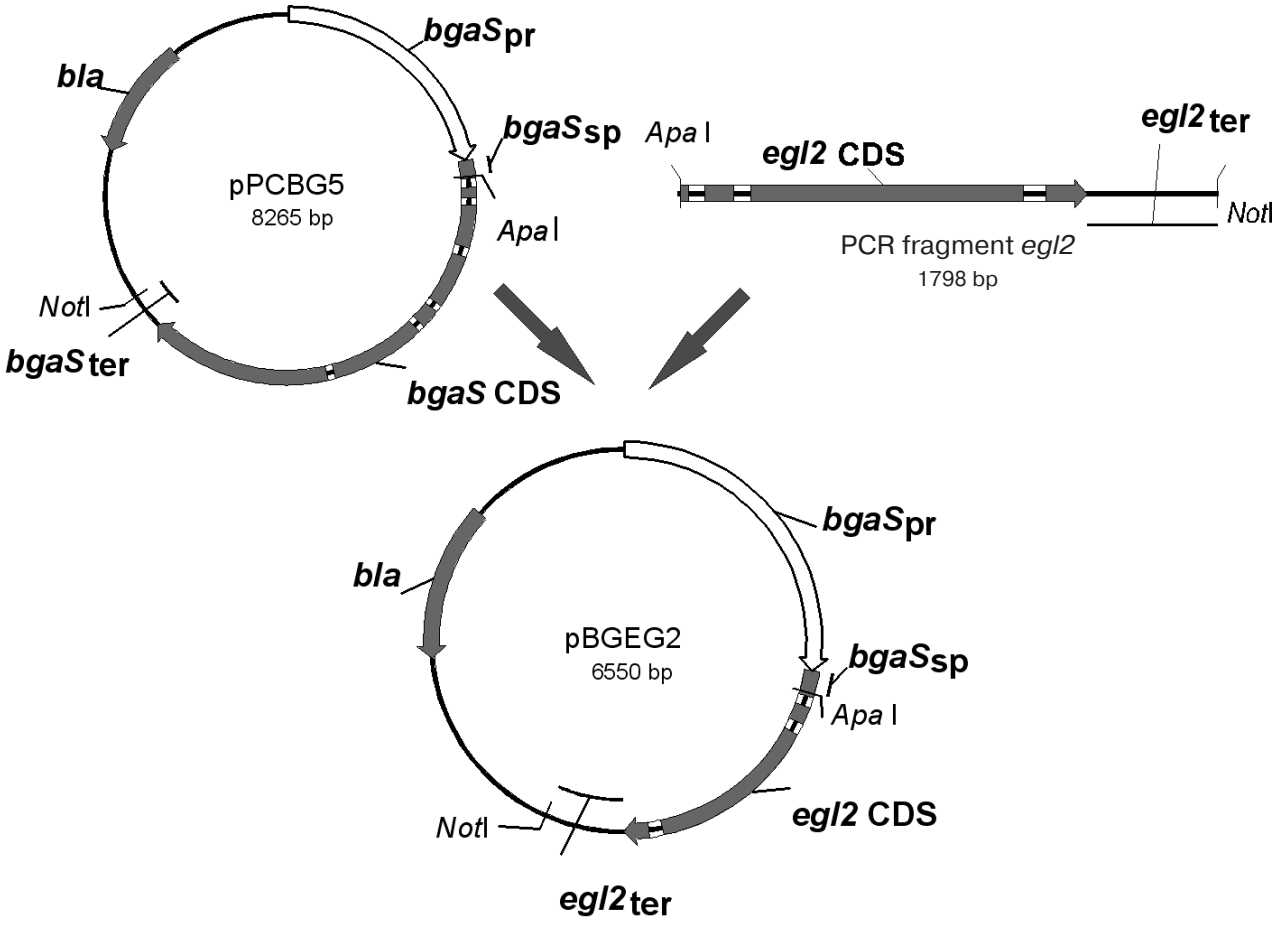

Fig. 4. Scheme of the pBGEG2 plasmid design. Genes and restriction sites are shown.

5 present in mycelial fungi belong to the class of acidic mesophilic cellulases and have pH optimum in the pH range 3-6 and temperature optimum of activity in the range of 50-65°C [13, 14]. Our preparation of Eg12p has maximal activity at pH 3.4 (Fig. 6) and temperature 60°C (Fig. 7). At pH values above 4.6, Eg12p loses up to 50% of its activity, which makes possible its application in technological processes that take place at pH from 3 to 4.5. Our enzyme exhibited over 80% of activity in the range of temperature 54-65°C, which allows its application in industrial technologies at high temperatures. The enzyme lost over 50% of its activity at temperatures above 68°C.

β-Galactosidase and endoglucanase activities after transformant growth

Strain	Activity, IU	
	β-galactosidase	endoglucanase
PCA-10 (recipient)	68.4	1.0
EG2-4	18.4	43.0
EG2-11	25.0	34.0
EG2-17	27.3	37.0

Differential scanning calorimetry (DSC). There are no data available in the literature concerning investigation of endoglucanase thermal stability by DSC. We showed by DSC analysis that the recombinant Eg12p irreversibly denatured at 63°C (Fig. 7). Changes in the protein tertiary structure take place already at temperatures above 50°C. At the same temperatures increased Eg12p activity was observed, and the temperature optimum for enzyme activity was observed at 60°C. Analysis of our data suggests that melting of the protein tertiary structure results in enzyme inactivation, which is also confirmed by the thermostability profile at 50°C (Fig. 8). This can be explained by inability of the protein active center to be involved in the catalytic cycle after the loss of tertiary spatial structure.

Kinetic parameters. Values of K_m and V_{max} upon CM-cellulose hydrolysis were 10.28 g/liter and 0.26 μmol/sec per mg, respectively, while the relative parameter V_{max}/K_m

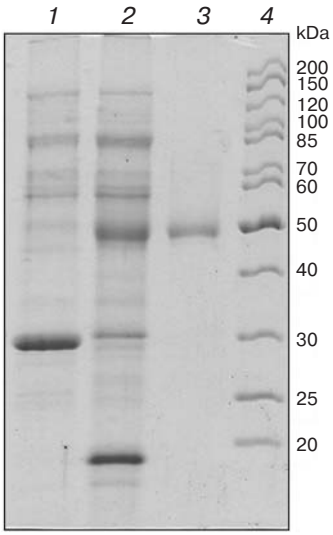

Fig. 5. SDS-PAGE of *P. canescens* recipient strain PCA-10 (*1*), secreted proteins of recombinant strain EGL2-4 (*2*), homogeneous Eg12p (*3*), and protein marker (*4*).

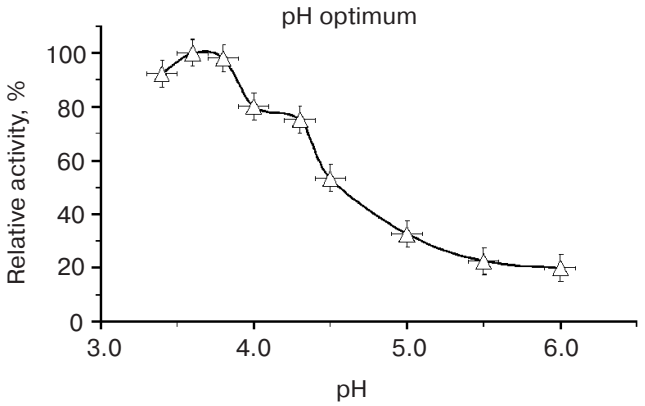

Fig. 6. The pH dependence of Eg12p activity at 40°C in 0.1 M universal buffer.

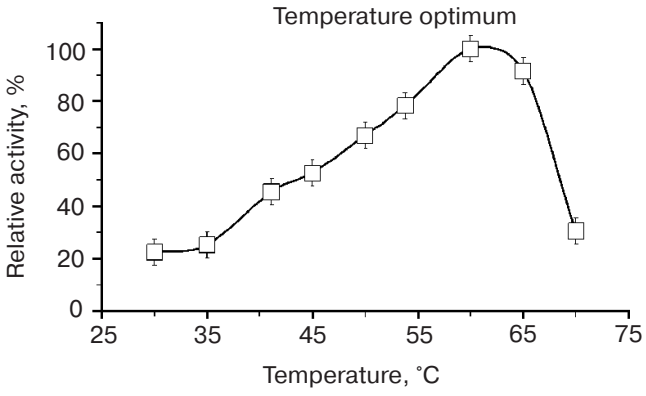

Fig. 7. Temperature dependence of Eg12p endoglucanase activity in 0.1 M Na-acetate buffer, pH 4.5.

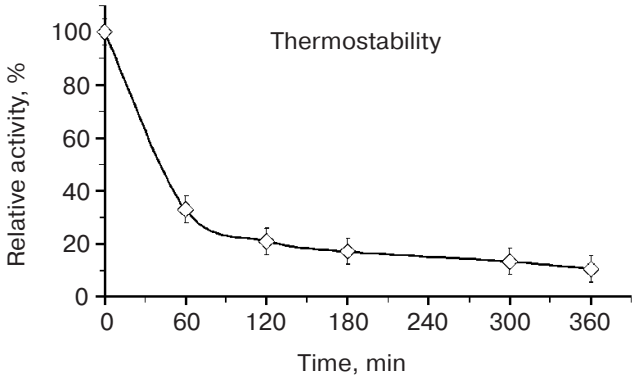

Fig. 8. Thermostability profile of Eg12p at 50°C in 0.1 M Na-acetate buffer, pH 4.5.

was $2.56 \cdot 10^{-5}$. The available literature does not contain any data concerning kinetic parameters of fungal and recombinant endoglucanases of this family.

Thus, this is the first complex study of biochemical and physicochemical properties of endoglucanase of glycosyl hydrolase family 5 isolated from the *P. canescens* culture liquid. As a result, homogeneous enzyme was obtained. The studied Eg12p appeared to be comparable in its biochemical and physicochemical properties with other members of this family.

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REFERENCES

1. Sinitsyn, A. P., Gusakov, A. V., and Chernoglazov, V. M. (1995) *Bioconversion of Lignocellulose Materials* [in Russian], MGU Publishing House, Moscow.
2. Clarke, A. J. (1997) *Biodegradation of Cellulose. Enzymology and Biotechnology*, Technomic Publisher Co., Lancaster.
3. Aleksenko, A. Y., Makarova, N. A., Nikolaev, I. V., and Clutterbuck, A. J. (1995) *Curr. Genet.*, **28**, 474-478.

4. Nikolaev, I. V., Bekker, O. B., Serebryanyi, V. A., Chulkin, A. M., and Vinetskii, Yu. P. (1999) *Biotekhnologiya*, **3**, 3-13.
5. Nikolaev, I. V., and Vinetskii, Yu. P. (1998) *Biochemistry (Moscow)*, **63**, 1294-1298.
6. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
7. Somogyi, M. (1952) *J. Biol. Chem.*, **195**, 19-28.
8. Nelson, N. (1944) *J. Biol. Chem.*, **153**, 375-380.
9. Varfolomeev, S. D., and Gurevich, K. G. (1999) *Biokinetics: Practical Course* [in Russian], FIAR-PRESS Publishing House, Moscow.
10. Siebert, P. D., Chenchik, A., Kellogg, D. E., Luk'yanov, K. A., and Luk'yanov, S. A. (1995) *Nucleic Acids Res.*, **23**, 1087-1088.
11. Rambosek, J., and Leach, J. (1987) *Crit. Rev. Biotechnol.*, **6**, 357-393.
12. Unkles, S. E., Campbell, E. I., Punt, P. J., Hawker, K. L., Contreras, R., Hawkins, A. R., van den Hondel, C. A., and Kinghorn, J. R. (1992) *Gene*, **111**, 149-155.
13. Schulein, M. (1997) *J. Biotechnol.*, **57**, 71-81.
14. Takashima, S., Takashima, S., Iikura, H., Nakamura, A., Hidaka, M., Masaki, H., and Uozumi, T. (1998) *J. Biotechnol.*, **65**, 163-171.