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Cellulase Complex of the Fungus *Chrysosporium lucknowense*: Isolation and Characterization of Endoglucanases and Cellobiohydrolases

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Abstract—Using different chromatographic techniques, eight cellulolytic enzymes were isolated from the culture broth of a mutant strain of *Chrysosporium lucknowense*: six endoglucanases (EG: 25 kD, p*I* 4.0; 28 kD, p*I* 5.7; 44 kD, p*I* 6.0; 47 kD, p*I* 5.7; 51 kD, p*I* 4.8; 60 kD, p*I* 3.7) and two cellobiohydrolases (CBH I, 65 kD, p*I* 4.5; CBH II, 42 kD, p*I* 4.2). Some of the isolated cellulases were classified into known families of glycoside hydrolases: Cel6A (CBH II), Cel7A (CBH I), Cel12A (EG28), Cel45A (EG25). It was shown that EG44 and EG51 are two different forms of one enzyme. EG44 seems to be a catalytic module of an intact EG51 without a cellulose-binding module. All the enzymes had pH optimum of activity in the acidic range (at pH 4.5-6.0), whereas EG25 and EG47 retained 55-60% of the maximum activity at pH 8.5. Substrate specificity of the purified cellulases against carboxymethylcellulose (CMC), β-glucan, Avicel, xylan, xyloglucan, laminarin, and *p*-nitrophenyl-β-D-cellobioside was studied. EG44 and EG51 were characterized by the highest CMCase activity (59 and 52 U/mg protein). EG28 had the lowest CMCase activity (11 U/mg) amongst the endoglucanases; however, this enzyme displayed the highest activity against β-glucan (125 U/mg). Only EG51 and CBH I were characterized by high adsorption ability on Avicel cellulose (98-99%). Kinetics of Avicel hydrolysis by the isolated cellulases in the presence of purified β-glucosidase from *Aspergillus japonicus* was studied. The hydrolytic efficiency of cellulases (estimated as glucose yield after a 7-day reaction) decreased in the following order: CBH I, EG60, CBH II, EG51, EG47, EG25, EG28, EG44.

Key words: cellulase, endoglucanase, cellobiohydrolase, chromatography, adsorption, saccharification

The cellulolytic enzymes, responsible for biodegradation of cellulose, the most widespread biopolymer on Earth, occupy the central position in the organic carbon cycle [1]. The main cellulase-producing microorganisms are fungi, soft and brown rot pathogens, and also different species of aerobic and anaerobic bacteria. Enzymes of the cellulase complex include endo-1,4-β-glucanase (EC 3.2.1.4), exo-cellobiohydrolase (EC 3.2.1.91), and also β-glucosidase (EC 3.2.1.21) [1, 2]. Recently, the cellulases have been increasingly used in the textile, pulp and paper, food, and other industries [3]. Therefore, the search for new cellulases and investigation of their properties are of great importance from both scientific and practical points of view.

The mycelial fungus *Chrysosporium lucknowense*, classified as an ascomycete, was isolated from Far East alkaline soil at the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences. Mutant strains characterized by increased secretion level of cellulases and hemicellulases were further obtained using classic mutagenesis. The enzyme preparations from this fungus exhibited high efficiency in the treatment of cotton textiles [4]. However, data on the composition and functional characteristics of a multienzyme complex from *C. lucknowense* as well as information about the enzymes produced by other *Chrysosporium* fungi are scarce in literature.

The aim of the present work was the isolation and investigation of properties of individual cellulases from the multienzyme complex produced by a mutant strain of the fungus *C. lucknowense*.

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MATERIALS AND METHODS

Enzymes. A laboratory enzyme preparation obtained at the Institute of Biochemistry and Physiology of Microorganisms and based on a mutant strain of *C. lucknowense* was used in this work. The preparation was an ultrafiltrate of the fungus culture broth. For enzymatic hydrolysis of microcrystalline cellulose (MCC) by purified *C. lucknowense* enzymes, the purified β-glucosidase from *Aspergillus japonicus* supplied by NPO Biotekhnika with a specific activity of 50 U/mg (40°C, pH 5.0) was used.

Substrates. Medium viscosity carboxymethylcellulose (CMC), birch xylan, laminarin, *p*-nitrophenyl-β-D-cellobioside (*p*-NP-G_2) were purchased from Sigma (USA); xyloglucan and barley β-glucan were from Megazyme (Australia), MCC (Avicel PH 105) was supplied by Serva (Germany).

Determination of enzyme activities. The enzyme activities against polysaccharide substrates were determined according to the initial rates of reducing sugar formation using the Somogyi–Nelson technique [5, 6]. The activity unit was the amount of enzyme resulting in the formation of 1 μmol of reducing sugars (RS) from the corresponding substrate (0.5%) in 1 min at pH 5.0 (0.1 M Na-acetate buffer) and 50°C [2]. The endoglucanase activity was also determined by a viscometric technique [2] using 0.5% CMC (40°C, pH 5.0).

The activity against *p*-NP-G_2 was determined at pH 5.0 and 40°C. An aliquot (0.05 ml) of the substrate stock solution (10 mM) was mixed with 0.85 ml of 0.1 M Na-acetate buffer. The mixture was heated at 40°C for 5 min and the enzymatic reaction was initiated by addition of 0.1 ml of enzyme solution, which was suitably diluted and heated the same way. The reaction was stopped exactly after 10 min incubation at 40°C by adding 0.5 ml of 1 M Na_2CO_3 solution. After that, the absorption at 400 nm was measured against the reference cuvette containing the substrate solution, prepared according to the above procedure (0.1 ml of acetate buffer was added instead of the enzyme). The activity unit was the amount of enzyme resulting in the formation of 1 μmol of *p*-nitrophenol for 1 min under the described conditions.

Protein content in the samples was assayed either by the Lowry method using bovine serum albumin as a standard or by the absorption at 280 nm [7].

Isolation and purification of individual enzymes from the cellulase complex. The general scheme of enzyme purification is presented in Fig. 1.

Anion exchange chromatography on a Source 15Q column (1.6 × 5 cm) using an FPLC system (Pharmacia, Sweden) was applied for the isolation of the enzymes. Enzyme preparation (an ultrafiltrate from culture broth) was desalted on a column with Acrylex P2 (Reanal, Hungary) in 20 mM Bis-Tris-HCl buffer, pH 6.6. A sample containing 150 mg of the protein was applied on a Source 15Q column equilibrated with the same buffer. The column was washed with a starting buffer, and the bound protein was eluted with a NaCl gradient at a flow rate of 5 ml/min (the gradient volume was 400 ml). The resulting chromatogram is shown in Fig. 2. In total, three purification runs were performed, where the fractions

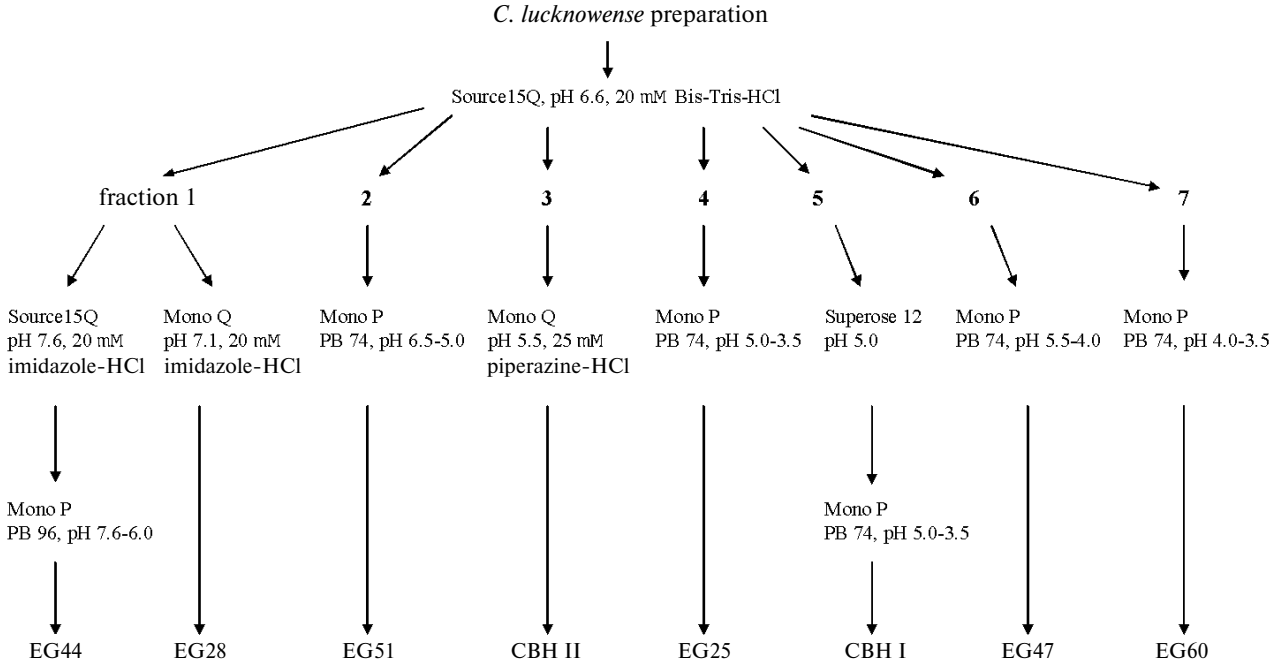

Fig. 1. Strategy for the isolation of cellulases from *C. lucknowense*.

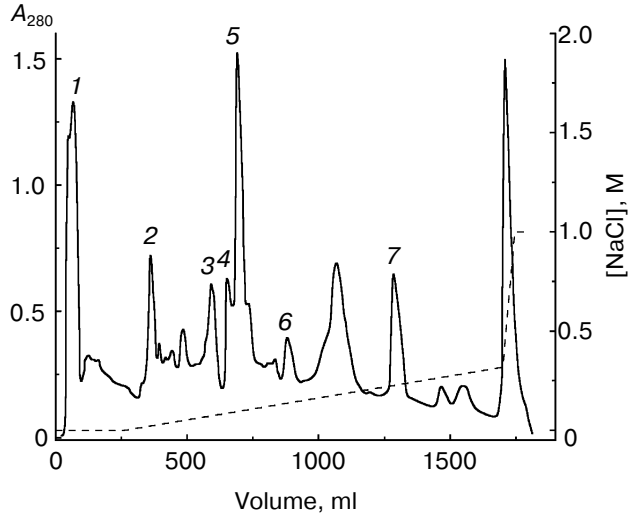

Fig. 2. Fractioning of the original multienzyme preparation on a Source 15Q column. On the auxiliary axis (to the right), salt concentration gradient is plotted. Fractions: *1*) EG44 and EG28; *2*) EG51; *3*) CBH II; *4*) EG25; *5*) CBH I; *6*) EG47; *7*) EG60.

with similar activity were pooled. All desalting procedures and buffer changes were subsequently performed on a column with Acrylex P2.

Protein fraction 1 (Fig. 2) which was not bound at the first purification step contained two endoglucanases with molecular masses 44 and 28 kD (EG44 and EG28). To isolate EG44, the fraction was transferred into 20 mM imidazole-HCl buffer, pH 7.6, and applied on a Source 15Q column, equilibrated at pH 7.6. EG44 was eluted by the salt gradient, deionized, and then applied to chromatofocusing on a Mono P column in pH gradient of 7.6-6.0 (using Polybuffer PB 96), which produced homogeneous enzyme. To isolate EG28, unbound protein fraction was transferred into 20 mM imidazole-HCl buffer, pH 7.1, and applied on an anion-exchange Mono Q column equilibrated with the same buffer. Most of the proteins did not bind to the column at this pH. Homogeneous EG28 was eluted at NaCl concentration of 0.05 M.

Endoglucanase with a molecular mass of 51 kD (EG51) was found in fraction 2 eluted by the NaCl gradient in the first purification step. The homogeneous enzyme was isolated by chromatofocusing on a Mono P column in pH gradient of 6.5-5.0 using Polybuffer PB 74 (preliminarily the fraction was transferred into 25 mM Bis-Tris-HCl buffer, pH 6.5).

Endoglucanases with molecular masses of 25, 47, and 60 kD (EG25, EG47, and EG60) were isolated from the corresponding protein fractions (Fig. 1) by chromatofocusing under different conditions. EG25 was isolated in a pH gradient of 5.0-3.5. To perform chromatofocusing of EG47 and EG60, pH gradients of 5.5-4.0 and 4.0-3.5 were used, respectively. The protein fractions containing these endoglucanases were transferred into 25 mM piperazine-HCl buffer (EG25 and EG47) or methylpiperazine-HCl buffer (EG60) with the corresponding pH values.

Cellobiohydrolase I (CBH I, 65 kD) from fraction 5 after Source 15Q was further purified by gel permeation chromatography on a Superose 12 column (Pharmacia). The column was equilibrated with 0.1 M Na-acetate buffer, pH 5.0. The protein was eluted at a flow rate of 0.3 ml/min. To remove trace amounts of admixtures, the CBH I containing fraction was additionally subjected to chromatofocusing on Mono P column under the same conditions as described for the isolation of EG25.

CBH II (43 kD) was isolated by anion-exchange chromatography. Fraction 3 obtained after the first purification step was applied on a Mono Q column equilibrated with 25 mM piperazine-HCl buffer, pH 5.5. The homogeneous protein emerged at 0.1 M NaCl concentration in a salt gradient.

Determination of biochemical characteristics of the individual enzymes. Analytical isoelectrofocusing of proteins was performed on a Model 111 Cell unit (Bio-Rad, USA) according to the instructions. Protein electrophoresis was performed in 12% polyacrylamide gel in the presence of SDS on Mini Protean unit (Bio-Rad) according to the instructions. Protein bands in the gel were stained with Coomassie G-250.

Determination of adsorption properties of the enzymes. Adsorption of the purified cellulases on MCC (Avicel PH 105) was studied at 4°C and pH 5.0. Cellulose suspension (5%) in enzyme solution (0.2 mg/ml) was incubated in a thermostatic water bath at 4°C while stirring for 30 min on a magnetic stirrer. After that, the mixture was centrifuged at 15,000 rpm for 3 min; the residual protein concentration in the supernatant was determined according to Lowry et al. [7]. The results were expressed as a percentage of the adsorbed protein (compared to initial value).

Kinetic studies of MCC hydrolysis. Hydrolysis of 0.5% MCC catalyzed by the purified cellulases was performed for 7 days at 40°C and pH 5.0. An amount of substrate (10 mg) was mixed with 1 ml of 0.1 M Na-acetate buffer, pH 5.0, containing 1 mM of NaN_3 to prevent microbial growth, in a 2 ml vial. The vial was incubated in a thermostatic water bath equipped with a magnetic stirrer. Enzyme solution (1 ml) diluted to the required concentration was added, and the mixture was stirred. Equal protein concentrations (0.1 mg/ml) were used for all enzyme preparations. To convert cellooligosaccharides (which are soluble products of cellulose hydrolysis catalyzed by endoglucanases and cellobiohydrolases) into a final hydrolysis product (glucose) and thus simplify the analysis of hydrolyzate, highly purified β-glucosidase from *A. japonicus* (0.2 U/ml) was added to the reaction mixture. Aliquots of the suspension (0.1 ml) were collect-

ed from the reaction mixture and centrifuged after fixed time intervals. Glucose concentration in the supernatant was determined by the glucose oxidase-peroxidase technique [2] using a Fotoglukoza analysis kit produced by Impact Ltd. (Russia).

Low molecular weight products of polysaccharide hydrolysis were determined by HPLC on a column with bonded amino phase [2]. A Chromatography Workstation 700 HPLC system (Bio-Rad) with a refractometric detector was used. A mixture of acetonitrile with water (7 : 3) was used as the eluent. The chromatography was performed at 1 ml/min flow rate.

RESULTS

Isolation of cellulases. The scheme of *C. lucknowense* cellulase purification is illustrated in Fig. 1. Using anion-exchange chromatography on a Source 15Q column (Fig. 2) followed by anion-exchange chromatography on Mono Q, gel filtration on Superose 12, and chromatofocusing on Mono P, eight enzymes of the cellulase complex were isolated: six endoglucanases and two cellobiohydrolases. Total protein content (estimated by A_{280}), CMCase activity (for endoglucanases), and activities against Avicel (MCC) and *p*-NP-G_2 (for cellobiohydrolases) were monitored in the protein fractions during purification. The first purification step included anion-exchange chromatography at pH 6.6. In the initial experiments, the column was equilibrated at pH 6.5; however, under these conditions EG51 was detected in the unbound fraction, which significantly complicated the enzyme isolation procedure. Increasing pH to 6.6 produced EG51 in the initial fraction of salt gradient with content of the target protein up to 70%. Using subsequent chromatofocusing in a pH gradient of 6.5-5.0, the homogeneous enzyme was isolated.

The unbound fraction after anion-exchange chromatography on Source 15Q contained EG44 and EG28. Both enzymes could be isolated by the chromatofocusing technique; however, a more convenient approach for the purification of EG28 was anion-exchange chromatography on a Mono Q column at pH 7.1. Under these conditions, most of the target protein bound to the column.

EG25, EG47, EG60, CBH I, and CBH II were isolated from the corresponding protein fractions after Source 15Q by chromatofocusing, gel filtration, or anion-exchange chromatography (Fig. 1).

It should be noted that CBH I from *C. lucknowense* exists in two forms (65 and 52 kD) [8]. The low molecular weight (truncated) form (52 kD) is a catalytic module of the intact CBH I (65 kD), which lacks a cellulose-binding module (CBM) and a glycosylated linker. Earlier, these two CBH I forms have been isolated and thoroughly characterized by us [8]. In the present work, we isolated only the intact CBH I, aiming to compare it to endoglucanases and CBH II, which were isolated and described for the first time.

Major properties and classification of the purified cellulases. Eight enzymes involved in the degradation of cellulose were isolated as a result of the fractionation of *C. lucknowense* multienzyme preparation. Using SDS-PAGE and isoelectrofocusing, the molecular weights and isoelectric points for the enzymes were determined (Figs. 3 and 4 and Table 1). All the mentioned enzymes were virtually homogeneous and appeared as one band on the corresponding gel electrophoregrams, with the exception of EG44 and EG47, which also contained minor bands along with the major bands after the electrofocusing. Based on the calculated chromatographic peak areas cor-

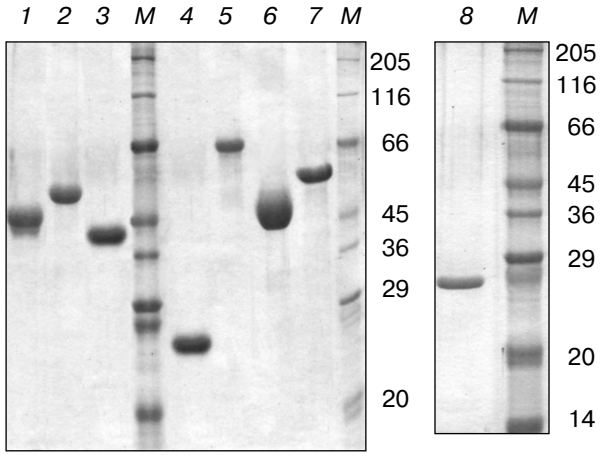

Fig. 3. SDS-PAGE of the isolated enzymes: EG44 (*1*), EG51 (*2*), CBH II (*3*), EG25 (*4*), CBH I (*5*), EG47 (*6*), EG60 (*7*), EG28 (*8*). In the middle and to the right, protein markers (M) with different molecular weights (in kD) are shown.

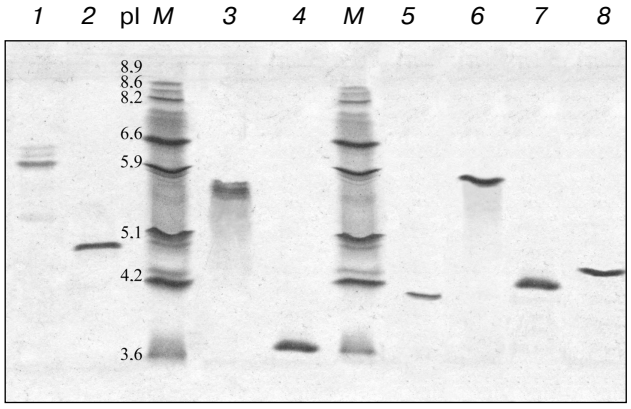

Fig. 4. Isoelectrofocusing of the isolated enzymes: EG44 (*1*), EG51 (*2*), EG47 (*3*), EG60 (*4*), EG25 (*5*), EG28 (*6*), CBH II (*7*), CBH I (*8*). In the middle, protein markers (M) with different p*I* values are shown.

Table 1. Biochemical and physicochemical characteristics of the isolated enzymes

Enzyme	pI	Family of glycoside hydrolases	Presence of CBM	Content in the sample, %	Adsorption on MCC, %	CMC		β-Glucan	
						K_m, g/liter	k_{cat}, sec^{-1}	K_m, g/liter	k_{cat}, sec^{-1}
EG25	4.0	45	—	2	20	5.4	15	3.0	3.8
EG28	5.7	12	—	2	22	11	19	0.12	64
EG44	6.0	n.d.	—	<1	10	1.3	54	1.14	76
EG47	5.7	n.d.	—	7	29	6.8	27	0.46	16
EG51	4.8	n.d.	+	6	98	2.4	54	1.8	84
EG60	3.7	n.d.	—	1	14	4.6	22	0.17	18
CBH I (65 kD)	4.5	7	+	15	99	n.d.	n.d.	n.d.	n.d.
CBH II (43 kD)	4.2	6	—	2	32	n.d.	n.d.	n.d.	n.d.

Note: CBM, cellulose-binding module; n.d., not determined.

Table 2. Specific activities of the endoglucanases and cellobiohydrolases against various substrates (U/mg protein)

Enzyme	CMC (RS)	CMC (viscometry)	β-Glucan	Avicel (MCC)	Xyloglucan	Laminarin	Xylan	*p*-NP-G_2
EG25	17	20	5.3	0.05	1.9	0	0	0
EG28	11	19	125	0.02	16	0	0.2	0
EG44	59	39	74	0.06	0	0.13	0.07	0
EG47	14	6	18	0.07	0	0	0.08	0
EG51	52	46	75	0.19	0	0.1	0.18	0
EG60	12	5	18	0.08	0	0	0	0.12
CBH I (65 kD)	0.2	0.01	0.1	0.19	0	0	0	0.02
CBH II (43 kD)	1.1	0.6	2.0	0.08	0	0	1.4	0

Note: RS, reducing sugars.

responding to the particular enzymes, the final yield (in mg) after the purification procedure, and also the intensity of corresponding protein bands on the electrophoregram of the original enzyme preparation, the content of each cellulase component in the original preparation was estimated (Table 1). It should be mentioned that these data are fairly approximate, but they indicate which of the individual cellulases from the multienzyme complex are produced by the fungus as major enzymes and which as the minor ones.

The substrate specificities of the isolated enzymes were determined using a number of polysaccharide substrates, as well as a synthetic chromogenic derivative of cellobiose (*p*-NP-G_2). The data are presented in Table 2. The first six enzymes shown in the table were characterized by a high level of CMCase activity and also by the ability to efficiently lower CMC viscosity. Based on these findings the enzymes were classified as endo-1,4-β-glucanases (EC 3.2.1.4) [2]. Taking into account the data acquired in our laboratory during the several years of cellulase investigation, typical values of CMCase activity for the purified fungal endoglucanases fall within the range of 10-50 U/mg of protein. EG44 and EG51 isolated from *C. lucknowense* enzyme preparation had specific CMCase activities of 59 and 52 U/mg, which indicates extremely high endoglucanase activity. EG28 had the lowest CMCase activity among the isolated endoglucanases (11 U/mg), but it exhibited an extraordinarily high specific activity against β-glucan (125 U/mg). Since β-glucan is a linear polysaccharide formed from glucose residues bound by β-1,4- and β-1,3-glucoside bonds, this enzyme could be classified as β-(1,3;1,4)-glucanase. However, since EG28 completely lacked activity against laminarin (β-1,3-glucan), it is obvious that the enzyme is strictly specific to β-1,4-bonds.

The exceptionally low activity against CMC combined with a high specific activity against Avicel cellulose and an appreciable activity against *p*-NP-G_2 allowed

unambiguous classification of the 65 kD enzyme as CBH I [8].

Certain difficulties were associated with classification of the 43 kD cellulase. This enzyme exhibited relatively low CMCase and β-glucanase activities for it to be classified as an endoglucanase, but it was characterized by a significant Avicelase activity, which is a characteristic feature for cellobiohydrolases. Only after peptide analysis and sequencing of the gene encoding this enzyme (see below) it could be unambiguously classified as CBH II.

Some of the purified cellulases also had additional activities against the other polysaccharide substrates (Table 2). It is worth mentioning the ability of EG25 and EG28 to hydrolyze xyloglucan, i.e., β-1,4-glucan containing xylose residues as substituents in the side chain. This property is not often encountered among endoglucanases. The same enzymes, compared to other endoglucanases, were characterized by higher activity during CMC hydrolysis as detected by viscometric technique, compared to CMCase activity determined by the formation of reducing sugars. This is an indication of less ordered action towards the polymeric substrate. With regard to the ability of certain isolated cellulases to hydrolyze laminarin and xylan, the observed specific activities were fairly low, which could be due to trace amounts of laminarinase or xylanase, or indicate a property specific for this particular enzyme.

The isolated cellulases were subjected to either chemical (BrCN) or enzymatic (trypsin) degradation with subsequent chromatographic separation and sequencing of the resulting peptides [8]. Based on the amino acid sequences, PCR primers for a number of the isolated enzymes (EG25, EG28, CBH I, CBH II) were synthesized, corresponding gene fragments were obtained, and, eventually, the full nucleotide sequences for the genes encoding these enzymes were determined. The genetic information was used to calculate the amino acid sequences for EG25, EG28, CBH I, and CBH II. Known homologous cellulases were found in the SWISS-PROT database. The detailed structural analysis of the peptides, genes, amino acid sequences, and their homology with known enzymes is beyond the scope of the present investigation (these data and their comprehensive analysis for CBH I are presented in [8]). Here it should be mentioned that analysis carried out for the four enzymes allowed not only confirming the correct classification with respect to enzyme action on the polymeric substrate (such as endoglucanases and cellobiohydrolases) but also establishing their affiliation to a particular family of glycoside hydrolases [9, 10] (Table 1). Therefore, the affiliation of EG25, EG28, CBH I, and CBH II to families 45, 12, 7, and 6 was determined, i.e., according to the classification proposed by Henrissat et al. [10] these enzymes should be referred to as Cel45A, Cel12A, Cel7A, and Cel6A, respectively (see also http://afmb.cnrs-mrs.fr/CAZY/). Moreover, the analysis of both complete gene and amino acid sequences of encoded proteins revealed that among the four mentioned proteins only CBH I contains a cellulose-binding module (CBM) in its molecule, which is a characteristic structural feature for most fungal cellulases [11].

Tryptic digestion of the gel bands corresponding to the particular enzymes after SDS-PAGE, followed by peptide extraction from the gel and subsequent analysis by mass-spectrometry, was performed [12] (at the Proteome Research Group of the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences). Analysis of the mass-spectra performed using MASCOT software (http://www.matrixscience.com) in the NCBI database (USA) did not reveal any identity of peptides from *C. lucknowense* protein tryptic digests to those from known enzymes. However, the data allowed unambiguous identification of EG44 and EG51 as two different forms of the same enzyme, since mass-spectra of the tryptic digests of these enzymes were practically identical. This conclusion is indirectly confirmed by similar specific activities of these endoglucanases against soluble polymeric substrates (CMC, β-glucan, laminarin; see Table 2), and also by similar pH dependences for the activities of these enzymes (Fig. 5).

The pH dependences for the activities of the isolated endoglucanases and cellobiohydrolases are shown in Fig. 5. All the enzymes exhibited maximal activity in acidic medium (optimal pH 4.5-6.0). It is important to note that two of the enzymes (EG25 and EG47) retained 55 and 60% of the maximal activity at pH 8.5. High activity at neutral and alkaline pH values is a feature that is rarely encountered among fungal cellulases.

The temperature optima for the activities of the purified *C. lucknowense* cellulases against CMC were within the range of 60-70°C (Fig. 6). The temperature dependence of EG28 activity is noteworthy. The enzyme exhibited more than 50% of the maximal activity at 30°C, while all the other cellulases displayed less than 25% of activity.

Investigation of thermostability demonstrated that all the isolated enzymes display high stability at 50°C and pH 5.0 and 7.0. Under these conditions, the purified cellulases retained more than 90% of activity for 5 h, except for EG28 at pH 7.0 (the enzyme lost 25% of its activity at pH 7.0, but it was stable at pH 5.0) and EG47. After 5 h incubation at pH 5.0 and pH 7.0, EG47 lost 40 and 20% of its initial activity, respectively.

Enzyme adsorption was evaluated at pH 5.0 and 4°C by determination of enzyme amount that was bound to MCC (Avicel PH 105) when the substrate was taken in a large excess (4 mg of protein per 1 g of the carrier). Only two enzymes (EG51 and CBH I) were characterized by high adsorption ability on MCC (protein adsorption extent was 98-99%). The adsorption extent did not exceed 32% for the other cellulases (Table 1). These data suggest that only EG51 and CBH I have cellulose-binding modules in their molecules [11]. The presence of a

CBM in CBH I and its absence in EG25, EG28, and CBH II molecules was proved by the analysis of genes encoding the corresponding proteins (see above). For the other enzymes (EG44, EG47, EG60) the conclusion about CBM absence (Table 1) is made based only on the data regarding their adsorption.

Kinetics of the purified endoglucanases. The kinetic parameters (K_m and k_{cat}) of the endoglucanases for CMC and β-glucan hydrolysis were determined based on the dependences of initial reaction rates on the substrate concentration (at 50°C and optimal pH). The found kinetic parameter values are presented in Table 1. Due to the inability to determine molecular weights of the substrates (both are polydisperse polymers), K_m was expressed in g/liter. EG44 and EG51 were characterized by the lowest K_m value (toward CMC) and highest k_{cat} (toward both substrates) compared to the other endoglucanases. It is noteworthy that similar or the same k_{cat} values, and similar K_m values for both substrates were obtained for both endoglucanases, which again confirms the conclusion that EG44 is a form of EG51. The K_m toward β-glucan in the case of EG28 was lower than K_m toward CMC by two orders of magnitude; at the same time k_{cat} toward β-glucan was more than three times higher. The kinetic parameters elucidate an extremely high β-glucanase activity of EG28.

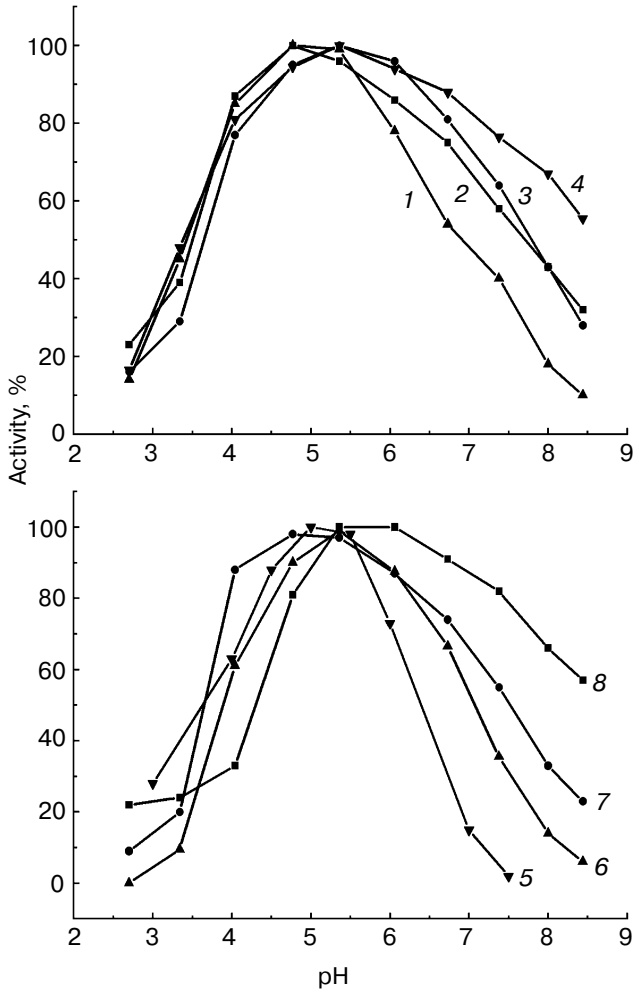

Fig. 5. Dependences of cellulase activity on pH: CBH II (*1*), EG51 (*2*), EG44 (*3*), EG25 (*4*), CBH I (*5*), EG28 (*6*), EG60 (*7*), and EG47 (*8*).

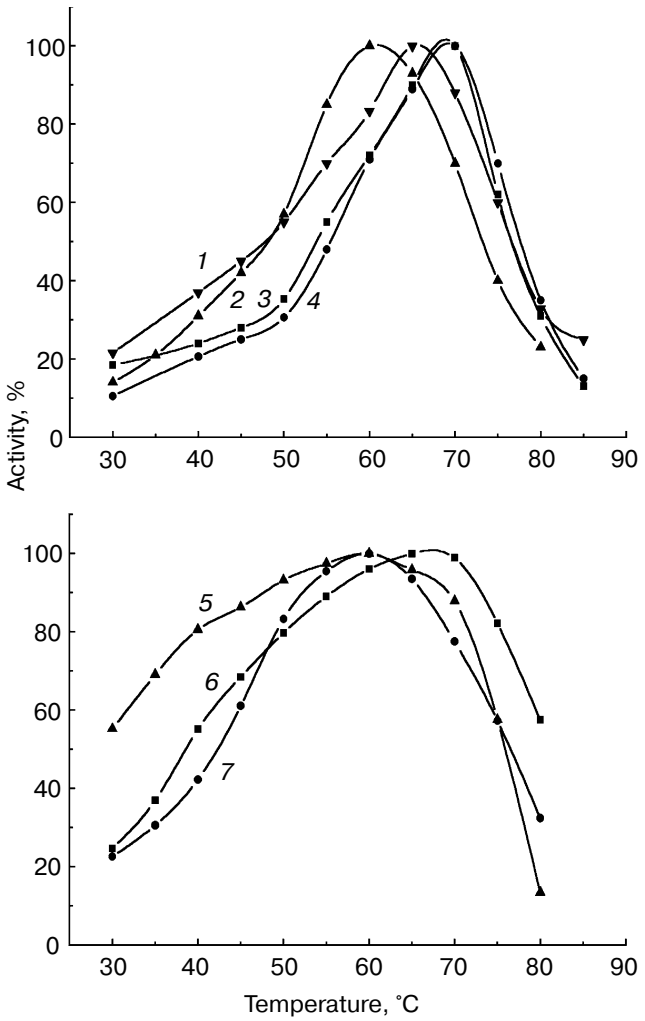

Fig. 6. Dependences of cellulase activity on temperature: EG25 (*1*), CBH II (*2*), EG44 (*3*), EG51 (*4*), EG28 (*5*), EG47 (*6*), EG60 (*7*).

The composition of low molecular weight products after complete hydrolysis of β-glucan by the purified endoglucanases was determined by HPLC using an amino phase column. The chromatograms are presented in Fig. 7. As seen from the comparison with the standard (glucose and oligosaccharides, chromatogram *7*), low molecular weight hydrolysis products were oligosaccharides with a polymerization degree between 2 and 4, and also glucose. The main hydrolysis products for EG44, EG51, EG28,

and EG47 were di- and trisaccharides, but also a significant amount of tetrasaccharide and relatively small amount of glucose were found. EG25 and EG60 resulted in a mixture of glucose with disaccharide and tetrasaccharide, where trisaccharide product was almost absent.

As expected, almost the only product of MCC hydrolysis by the purified CBH I and CBH II was cellobiose (>90%), and also trace amounts of glucose were found. Such product composition is typical for cellobiohydrolases [2, 13].

To compare the saccharification ability of the purified endoglucanases and cellobiohydrolases against insoluble cellulose, a long-term MCC hydrolysis was performed (Fig. 8). In this case, concentration of all cellulases in the reaction mixture was kept the same (0.1 mg/ml by protein). It is known that, as a rule, endoglucanases form a mixture of cellooligosaccharides (as soluble products of cellulose hydrolysis) whose concentration being usually too low to be quantitatively determined by the chromatographic techniques using a refractometer as a detector [2]. Therefore, the experiments on MCC hydrolysis for all the enzymes were performed in the presence of an excess of highly purified β-glucosidase (from *A. japonicus*), in order to convert formed cellobiose and other cellooligosaccharides into a final product, glucose, thus simplifying the analysis of the hydrolyzate. As expected, the highest efficiency of MCC hydrolysis was exhibited by CBH I, when glucose concentration after 7 days of reaction was 2.5 g/liter (MCC conversion degree was 45%). Significantly lower saccharification activity was found for EG51, CBH II, and EG60 (MCC conversion degree 19-23%). Endoglucanases with molecular weights 44, 28, 25, and 47 kD exhibited extremely low saccharification ability (substrate conversion degree did not exceed 12%).

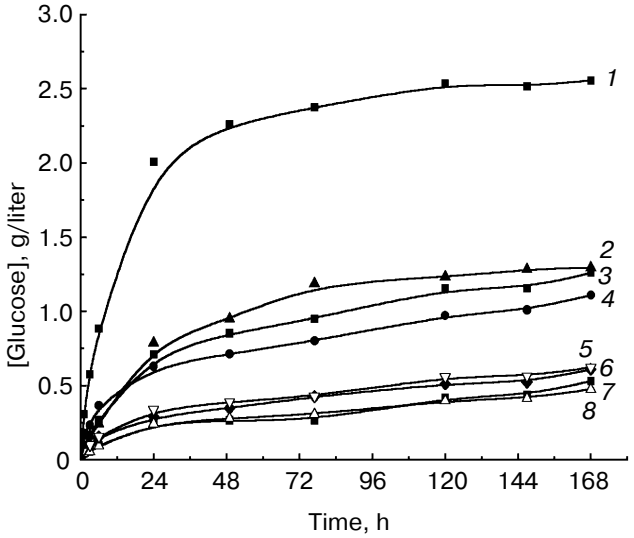

Fig. 8. Kinetic curves of glucose formation during MCC (0.5%) hydrolysis catalyzed by the cellulases from *C. lucknowense* (0.1 mg/ml) in the presence of purified β-glucosidase from *A. japonicus* (0.2 U/ml): *1*) CBH I; *2*) EG60; *3*) CBH II; *4*) EG51; *5*) EG47; *6*) EG25; *7*) EG28; *8*) EG44.

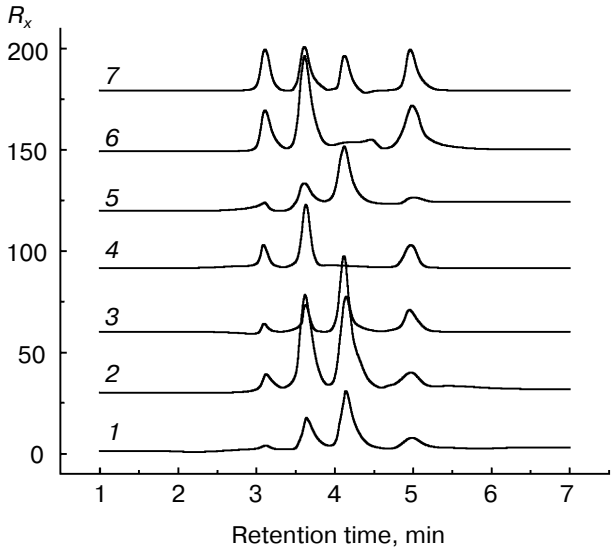

Fig. 7. Chromatographic separation of products of β-glucan hydrolysis catalyzed by the isolated endoglucanases: EG44 (*1*), EG51 (*2*), EG28 (*3*), EG25 (*4*), EG47 (*5*), EG60 (*6*); *7*) chromatographic standard (mixture of glucose, cellobiose, maltotriose, and maltotetraose).

DISCUSSION

Eight cellulolytic enzymes including six endoglucanases and two cellobiohydrolases were isolated from the culture broth of a mutant strain of *C. lucknowense*. The enzymes isolated in this study are similar to other fungal cellulases described in literature [1, 2, 14, 15] by their biochemical properties (molecular weight, p*I*), substrate specificity, and specific activity values. For instance, the most widely investigated cellulase complex from the fungus *Trichoderma reesei* consists of at least five endoglucanases, mainly two of which are secreted: EG I (Cel7B) and EG II (Cel5A); and also two cellobiohydrolases: CBH I (Cel7A) and CBH II (Cel6A) [1, 16]. The content of CBH I in *T. reesei* can reach 60% of the total secreted protein [17]. The fungus *Humicola insolens* also produces seven different cellulases (two of which are cellobiohydrolases), representing five families of glycoside hydrolases: 5, 6, 7, 12, and 45 [18]. A characteristic feature of almost all cellulases from *T. reesei* (except for Cel12A) and from *H. insolens* (five enzymes out of seven) is the presence of a CBM in their molecules, which is a special module enabling the absorption of enzymes on cellulose [11, 16, 18].

In our case, using various molecular techniques it was possible to establish homology between the four isolated enzymes from *C. lucknowense* and other known cellulases, whereby they were associated with the corresponding families of glycoside hydrolases: Cel6A (CBH II), Cel7A (CBH I), Cel12A (EG28), Cel45A (EG25). Additional studies are required for endoglucanases with molecular weights of 44, 47, 51, and 60 kD to determine their affiliation to the corresponding families. Using mass-spectrometry of the peptides obtained as the result of tryptic digestion of the proteins, it was possible to prove that EG44 and EG51 are two different forms of the same enzyme. Any identity between peptides obtained from other *C. lucknowense* cellulases was not found. Since both EG44 and EG51 exhibited very similar properties in the hydrolysis of soluble substrates (specific activities and kinetic parameters, composition of low molecular weight products, pH dependences of activities) and differed only by adsorption and Avicelase activity, it appears that the low molecular weight form (44 kD) is a catalytic module of a full size EG51 without the CBM. A very similar situation was observed in the case of CBH I from *C. lucknowense*, which was also represented by two forms, 52- and 65-kD. As we revealed earlier, the low molecular weight CBH I is formed as a result of proteolytic cleavage of the CBM and glycosylated linker from the full size CBH I (65 kD) during posttranslational modification [8].

Hence, *C. lucknowense* cellulase complex consists of at least five different endoglucanases (encoded by different genes) and two cellobiohydrolases. Among them, mostly secreted are CBH I, EG47, and EG51 (content of each enzyme in the original multienzyme preparation is 6-15% of the total protein amount). The total content of the other cellulases in the sample does not exceed 8% (Table 1).

Perhaps the most unexpected result was the fact that only in two of the cellulases isolated within this work (CBH I and EG51) a CBM was found (unlike many other cellulases described in literature, see above). It should be emphasized that the absence of a CBM in EG25 (Cel45A), EG28 (Cel12A), and CBH II (Cel6A) was confirmed by both the experiments on protein adsorption on MCC and analysis of the nucleotide sequences of the corresponding genes (and also amino acid sequences of proteins). Regarding EG47 and EG60, the absence of CBM in these enzymes was illustrated only by adsorption experiments and further research should clarify whether it is genetically pre-determined or CBM cleavage is taking place as the result of limited proteolysis of higher molecular weight forms of these enzymes that were not found.

All the isolated enzymes had pH optima of activities in acidic medium (at pH 4.5-6.0). Meanwhile, EG25 and EG47 retained 55-60% of maximum activity at pH 8.5. The high activity at neutral and alkaline pH values is not a specific feature for fungal cellulases; however, it is of great importance since cellulases are involved in biotechnological processes such as enzymatic treatment of cotton textiles and used as additives to detergents [3, 4]. In this case, sometimes it is required that the enzyme is efficiently functioning at room temperature. From this point of view, EG28 is also a fairly promising enzyme, since it exhibits high activity at moderate temperature (30°C, Fig. 6).

The comparison of saccharification ability of cellulases from *C. lucknowense* affecting MCC has revealed that (as expected) CBH I the most efficiently hydrolyzes the insoluble cellulose. The significantly lower efficiency of CBH II is not surprising, since this enzyme does not contain a CBM. It is known that the hydrolytic activity of CBM-lacking cellulases is remarkably lower when compared to full-size enzymes containing a CBM [8, 19]. Among endoglucanases, the highest saccharification activity was displayed by EG60 and EG51. The fact that the low molecular weight (44 kD) form of EG51, which was weakly adsorbed on cellulose and is lacking a CBM, exhibited the least efficiency in MCC hydrolysis is fully explainable. However, the high saccharification ability of the weakly adsorbing EG60 was unexpected. This fact requires further investigation. Another point of interest is a study of enzyme interaction within the cellulase complex in the hydrolysis of crystalline cellulose. A mutual increase in the cellulase action (synergism) during the degradation of highly organized cellulose forms is known and often observed [2, 20]. However, taking into the account, that *C. lucknowense* cellulase complex includes at least seven different cellulases, it is impossible to present the results on enzyme synergetic interaction within the framework of this study; thus, the data will be presented in another article.

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