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Isolation and Properties of Fungal β-Glucosidases

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Abstract—Using chromatography on different matrixes, three β-glucosidases (120, 116, and 70 kDa) were isolated from enzymatic complexes of the mycelial fungi *Aspergillus japonicus*, *Penicillium verruculosum*, and *Trichoderma reesei*, respectively. The enzymes were identified by MALDI-TOF mass-spectrometry. Substrate specificity, kinetic parameters for hydrolysis of specific substrates, ability to catalyze the transglucosidation reaction, dependence of the enzymatic activity on pH and temperature, stability of the enzymes at different temperatures, adsorption ability on insoluble cellulose, and the influence of glucose on catalytic properties of the enzymes were investigated. According to the substrate specificity, the enzymes were shown to belong to two groups: i) β-glucosidase of *A. japonicus* exhibiting high specific activity to the low molecular weight substrates cellobiose and *p*NPG (the specific activity towards cellobiose was higher than towards *p*NPG) and low activity towards polysaccharide substrates (β-glucan from barley and laminarin); ii) β-glucosidases from *P. verruculosum* and *T. reesei* exhibiting relatively high activity to polysaccharide substrates and lower activity to low molecular weight substrates (activity to cellobiose was lower than to *p*NPG).

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Key words: β-glucosidase, *Aspergillus japonicus*, *Penicillium verruculosum*, *Trichoderma reesei*, saccharification, transglucosidation, HPLC

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyzes hydrolytic cleavage of the β-glycoside bond. In terms of the substrate specificity, β-glucosidases can be divided into three types [1-5]: β-glucosidases (cellobiases), aryl-β-glucosidases, and β-glucosidases exhibiting wide substrate specificity towards the aglycone part of the molecule, i.e. hydrolyzing disaccharides, aryl glucosides, and corresponding oligosaccharides.

β-Glucosidases play a rather significant role in biotechnological and physiological processes [6-8]. During enzymatic hydrolysis of cellulose by cellulases, β-glucosidases hydrolyze cellobioses, increasing the yield of the final product and decreasing the concentration of cellobiose in the reaction mixture (cellobiose is a strong inhibitor of cellulolytic enzymes, especially cellobiohydrolases). Wide distribution of β-glucosidases in nature is explained by the necessity to release glucose as the source of carbon and energy from natural β-D-glucosides and low molecular weight cellooligosaccharides.

The most important producers of β-glucosidases are microscopic fungi. The world market of enzymatic preparations presents a broad spectrum of cellulases, most of which are prepared from enzymes produced by fungi of the genus *Trichoderma*. There are also preparations obtained using the fungi *Penicillium* and *Aspergillus* [9, 10]. However, there are no comparative studies of the properties of β-glucosidases secreted by these microorganisms.

The goal of the present study was isolation of β-glucosidases of *Aspergillus japonicus*, *Penicillium verruculosum*, and *Trichoderma reesei*, investigation of their properties as well as possibility of their employment for sac-

Abbreviations: CBM, cellulose-binding module; CMC, carboxymethylcellulose; MCC, microcrystalline cellulose; PD, polymerization degree; *p*NPG, *p*-nitrophenyl-β-D-glucopyranoside.

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charification of cellulose, and finding a β-glucosidase that could be used for this purpose with the most efficiency.

MATERIALS AND METHODS

Enzymatic preparations. Two dry enzymatic preparations were obtained using the strains *P. verruculosum* and *T. reesei* in the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino). The first exhibited β-glucosidase activity of 1166 U/g (towards *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG)) and 1000 U/g (towards cellobiose); the activity of the second preparation was 50 U/g (towards *p*NPG) and 15 U/g (towards cellobiose). Besides, a dissolved preparation from *A. japonicus* was used with the activity of 140 U/ml towards *p*NPG and 253 U/ml towards cellobiose.

Substrates. Enzymatic activities were determined using the following substrates: *p*NPG (Sigma, USA); polymeric substrates – sodium salt of carboxymethylcellulose (CMC) of medium viscosity, laminarin from the algae *Laminaria digitata*, and β-glucan from barley (Sigma); microcrystalline cellulose (MCC) Avicel PH 105 (Serva, Germany); oligosaccharides – cellotriose, cellotetraose, cellopentaose, cellohexaose, and β-gentiobiose (Sigma), laminaribiose and laminaritriose (Megazyme, Australia), α-sophorose (Serva), cellobiose (Merck, Germany); D-glucose (ICN, USA).

Isolation of β-glucosidases. β-Glucosidases were isolated from the enzymatic preparations in three steps: preliminary purification, anion-exchange chromatography, and hydrophobic chromatography. The preparations of *P. verruculosum* and *T. reesei* were dissolved in 0.02 M piperazine-HCl, pH 6.2, and the preparation of *A. japonicus* was diluted 2-fold with 0.02 M piperazine-HCl buffer, pH 5.5. Then the samples were desalted on an Acrylex P-2 column (Reanal, Hungary) at a flow rate of 1 ml/min using a liquid Econo-System chromatograph (BioRad, USA). Subsequent purification steps were performed using an FPLC system (Pharmacia, Sweden). A Source 15Q HR 16/5 column (Pharmacia) was used for anion-exchange chromatography. A sample containing 100 mg of protein was applied to the column equilibrated with 0.02 M piperazine-HCl, pH 6.2 (or 5.5 for the preparation of *A. japonicus*). The non-adsorbed fraction was pooled, and the bound proteins were eluted with 400 ml of a linear NaCl gradient at a flow rate of 5 ml/min. The buffer in the collected fractions was changed using a Biogel P4 column (BioRad). A Source 15 Isopropyl column (Pharmacia) was used for purification of β-glucosidase from *P. verruculosum* and *T. reesei* and a phenyl-Superose column (Pharmacia) for purification of β-glucosidase from *A. japonicus* (hydrophobic chromatography). The columns were equilibrated with 1.7 M $(NH_4)_2SO_4$ in 0.05 M sodium acetate buffer, pH 5.0. The bound proteins were eluted with a linearly decreasing ammonium sulfate gradient (200 ml, 2 ml/min in the case of Source 15 Isopropyl or 20 ml, 0.5 ml/min in the case of phenyl-Superose). The resulting fractions were desalted on a Sephadex G-25 column (Pharmacia) using 0.1 M sodium acetate buffer, pH 5.0 (flow rate, 1 ml/min).

Protein content in the samples was determined by the Lowry method using BSA as the standard [11] or spectrophotometrically at 280 nm.

Biochemical characteristics of β-glucosidases. Electrophoresis of proteins under denaturing conditions (in the presence of SDS) was performed in 12% polyacrylamide gel using a Mini Protean system (BioRad). Protein bands in gels were stained with Coomassie Brilliant Blue R-250 (Ferak, Germany). Protein mixture MW-SDS 70L (14-66 kDa), phosphorylase B (97 kDa), and β-galactosidase (116 kDa) (Sigma) were used as the standards.

MALDI-TOF mass-spectrometric analysis of trypsin hydrolysate of β-glucosidases [12-14]. A section of the SDS-polyacrylamide gel corresponding to the protein band of β-glucosidase was treated with trypsin [13] (genetically modified trypsin (Promega, USA) was used for protein sequencing: 5 ng/ml in 0.05 M NH_4HCO_3). The resulting peptides were extracted with 20% acetonitrile containing 0.1% trifluoroacetate and analyzed by MALDI-TOF mass-spectrometry using an Autoflex II mass spectrometer (Bruker Daltonics, Germany) in the laboratory of Physical Organic Chemistry (Chemical Faculty, Moscow State University). Tandem TOF-TOF mass-spectrometry of peptides was carried out in the Emanuel Institute of Biochemical Physics using an Ultraflex mass spectrometer (Bruker Daltonics). Based on the obtained peptide mass values, peptides of identical masses within glycoside hydrolases were searched for in NCBI or Swiss-Prot databases using the MASCOT program (www.matrixscience.com). Homological proteins were searched for using the BLAST2 program (http://cn.expasy.org/tools/ and http://afmb.cnrs-mrs.fr/CAZY) [15].

Assay for enzymatic activities. The activities towards polysaccharide substrates were determined by the initial rates of the formation of reducing sugars by the modified Somogyi–Nelson method [16, 17], as well as by the bicinchoninate method [18].

The activity towards pNPG was determined by the initial rate of the accumulation of the colored reaction product at pH 5.0 and 40°C. A thermostatted plastic tube containing 0.9 ml of 1 mM substrate solution in 0.1 M sodium acetate buffer (40°C) was supplemented with 0.1 ml of an enzyme solution. The reaction mixture was mixed and incubated for 10 min. The reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3. The intensity of the colored product was determined spectrophotometrically at 400 nm, making correction for the

absorption of the substrate, enzyme, and acetate buffer. The activity was calculated using differential molar absorption coefficient $\Delta\varepsilon_{400} = 18{,}300\ M^{-1}\cdot cm^{-1}$.

In the case of large number of samples obtained after the chromatography, the activity was estimated using 96-well ELISA plates. The wells of a plate were supplemented with 180 μl of *p*NPG solution (0.5 mM in 0.1 M sodium acetate buffer) using a multichannel pipette. After 10 min of incubation at 40°C, the wells were supplemented with 20 μl of enzyme solution, and the reaction mixture was mixed. After 15 min, the reaction was stopped by the addition of 100 μl of 1 M Na_2CO_3. The absorption of the resulting solution was measured at 405 nm using an Anthos Labtec HT2 microplate reader (BioRad).

All specific activities were expressed in international units per mg protein (one unit corresponds to the amount of enzyme hydrolyzing 1 μmol of glycoside bonds per minute).

The cellobiase activity was determined by the method of Sinitsyn et al. [19] (40°C, pH 5.0, 2.5 mM substrate).

Glucose concentration was determined by the glucose oxidase–peroxidase method [20] using a Fotoglyukoza kit (Impakt, Russia).

Determination of kinetic parameters of hydrolysis of specific substrates. The kinetic parameters (K_m, k_{cat}) of β-glucosidases were determined from the dependence of the initial rate of hydrolysis on concentration of the substrate (*p*NPG, cellobiose, laminarin, and β-glucan) by nonlinear regression using the Michaelis–Menten equation and the Origin 6.1 program. The inhibition constants (K_i) and the type of the inhibition of β-glucosidases by glucose were determined from the dependence of the initial rate of *p*NPG hydrolysis on the initial substrate concentration (0.1-4 mM) at different concentrations of glucose (1, 2, 5, and 10 mM) at 40°C and pH 5.0. The data were calculated in Lineweaver–Burk coordinates. The inhibition constants were determined from the dependence of the apparent Michaelis constant ($K_{m\ app}$) on the inhibitor concentration [21].

Determination of optimal conditions (pH and temperature) for activity of β-glucosidases. The dependence of the β-glucosidase activity on pH was studied in 0.1 M citrate phosphate buffer, pH 3.0-7.5, at 40°C. The dependence of the activity on temperature was investigated in 0.1 M sodium acetate buffer, pH 5.0, and the temperature varying from 30 to 85°C. *p*NPG was used as the substrate. The samples were incubated at different pH values or temperature for 10 min.

Determination of thermostability of β-glucosidases. Enzyme solution (1.5 ml) was incubated at different temperatures (30, 40, and 50°C) in sodium acetate buffer, pH 5.0. After selected time intervals, aliquots were taken from the tested solutions, and the enzymatic activity towards *p*NPG was measured under standard conditions at 40°C and pH 5.0.

Study of the adsorption ability of β-glucosidases. Adsorption of β-glucosidases on MCC was studied at 4°C and pH 5.0. A suspension of MCC (50 g/liter) in a solution of the enzyme (0.02 g/liter) was incubated in a thermostat at 4°C for 30 min under constant stirring. Then the mixture was centrifuged (15,000 rpm, 3 min), and the supernatant was assayed for the residual activity towards *p*NPG under standard conditions. The results were expressed as the percentage of the adsorbed protein (in relation to the original concentration).

Study of transglucosidation. Mono- and oligosaccharides formed during the transglucosidation reaction were determined by HPLC analysis using a Dionex Carbopack PA-20 anion-exchange column (3 mm × 15 cm × 5 μm; Dionex Corporation, USA) and an Agilent 1100 HPLC system (Agilent, USA) equipped with an amperometric detector. The sugars were eluted with 7.5 mM NaOH. D-Glucose, cellobiose, cellotriose, β-gentiobiose, laminaribiose, laminaritriose, and α-sophorose were used as the standards.

Complete hydrolysis of specific substrates. Solutions of cellobiose (0.68, 3.42, and 17.1 g/liter, 30°C, pH 5.0), β-glucan (5 g/liter, 50°C, pH 5.0), or laminarin (5 g/liter, 50°C, pH 5.0) were incubated in the presence of homogenous enzyme preparation for several days. During the hydrolysis, aliquots were taken from the reaction mixture, and glucose concentration was determined by the glucose oxidase–peroxidase method.

RESULTS AND DISCUSSION

Isolation of homogenous β-glucosidases. Original preparations were purified from non-protein admixtures (insoluble compounds, carbohydrates, pigments, etc.) and subjected to anion-exchange chromatography on a Source 15Q column at pH 6.2 (for preparations of *P. verruculosum* and *T. reesei*) or 5.5 (the preparation of *A. japonicus*). The β-glucosidase activity for the first two preparations was found in the fractions that passed through the column. For the preparation of *A. japonicus*, the activity was found in one of the fractions eluted with a NaCl gradient (0.2 M). From the results of SDS-PAGE, these fractions contained several proteins. To isolate β-glucosidases, the fractions were further purified using hydrophobic chromatography. The fractions containing β-glucosidases of *P. verruculosum* and *T. reesei* were purified on a Source 15 Isopropyl column, and the fraction containing β-glucosidase of *A. japonicus* was purified on phenyl-Superose. In the case of the first two preparations, the β-glucosidase activity was eluted with 1.1 and 0.8 M ammonium sulfate, respectively. In the case of the preparation of *A. japonicus*, the β-glucosidase activity was eluted with an ammonium sulfate gradient and was found in the fraction corresponding to the salt concentration of 0.7 M. The fractions were desalted by gel filtration on a Sephadex G-25 column. The pro-

cedure yielded homogenous (by SDS-PAGE) β-glucosidases of *P. verruculosum*, *T. reesei*, and *A. japonicus* of 116, 70, and 120 kDa, respectively.

Identification of β-glucosidases by mass-spectrometry analysis. After SDS-PAGE, protein bands corresponding to β-glucosidases were treated with trypsin, and the resulting hydrolyzates were assayed by MALDI-TOF mass-spectrometry. Analysis of the mass-spectra by the MASCOT program using the NCBI and Swiss-Prot databases showed that the β-glucosidases of *T. reesei* and *A. japonicus* are homologous to β-glycoside glucohydrolase of *T. reesei* and β-glucosidase of *A. kawachi*, respectively, belonging to the third family of glycoside hydrolases. For the third enzyme, no analogous proteins were found in the databases. To get information concerning the amino acid sequence and to identify six main peptides of the 116-kDa β-glucosidase of *P. verruculosum*, the enzyme was subjected to fragmentation with subsequent tandem (TOF-TOF) mass-spectrometry analysis. Using the BLAST2 program, the amino acid sequences that were similar to the peptides of β-glucosidase of *P. verruculosum* were found in the Swiss-Prot protein database. These sequences belonged to fungal β-glucosidases of the third family of glycoside hydrolases.

Substrate specificity of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei*. The specific activities of β-glucosidases towards different substrates are presented in Table 1. The activity of the β-glucosidase of *A. japonicus* was high towards the low molecular weight substrates (cellobiose and *p*NPG), being higher towards cellobiose than towards *p*NPG, and low towards the polysaccharide substrates (β-glucan from barley and laminarin). In contrast, the activities of the β-glucosidases of *P. verruculosum* and *T. reesei* were relatively high towards the polysaccharide substrates (β-glucan and laminarin) and lower towards the low molecular weight substrates (the activity towards cellobiose was lower than towards *p*NPG). None of the β-glucosidases exhibited activity towards CMC.

Thus, in terms of the substrate specificity, the isolated β-glucosidases can be divided into two groups: the β-glucosidase of *A. japonicus* is close to "true" cellobiase, and the β-glucosidases of *P. verruculosum* and *T. reesei* can be referred to enzymes exhibiting cellobiase and exo-1,3/1,4-β-glucosidase activity.

Such a division is supported by the following results. The influence of the polymerization degree (PD) of cellooligosaccharide substrates in the series cellobiose–cellohexaose on the enzyme activity was studied (Table 1). The activity of the β-glucosidase of *A. japonicus* (cellobiase) decreased with the increase in PD of the substrate, while the activity of the β-glucosidases of *P. verruculosum* and *T. reesei* (exo-β-glucosidases) did not depend on the PD of the substrate.

The specific activities of the isolated β-glucosidases towards α-sophorose, β-gentiobiose, and laminaribiose were also determined (Table 1). The β-glucosidase of *A. japonicus* exhibited a high activity towards β-gentiobiose and laminaribiose and showed a significantly lower activity towards α-sophorose. The β-glucosidases of *P. verruculosum* and *T. reesei* were virtually inactive towards β-

Table 1. Specific activities of β-glucosidases (U/mg)

Substrate	*A. japonicus*	*P. verruculosum*	*T. reesei*
	pH 5.0, 40°C		
*p*NPG	105	83	62
Cellobiose (1,4-β-bond)	124	54	21
Cellotriose	69	56	20
Cellotetraose	50	55	19
Cellopentaose	46	54	18
Cellohexaose	45	55	18
α-Sophorose (1,2-β-bond)	72	38	23
β-Gentiobiose (1,6-β-bond)	147	7	9
Laminaribiose (1,3-β-bond)	169	57	33
Laminaritriose	87	36	17
	pH 5.0, 50°C		
CMC	3	3	9
β-Glucan (β-1,3-1,4-bond)	9	52	39
Laminarin (β-1,3-bond)	32	102	81

Table 2. Kinetic parameters for hydrolysis catalyzed by β-glucosidases

Substrate	*A. japonicus*		*P. verruculosum*		*T. reesei*	
	K_m	k_{cat}, sec^{-1}	K_m	k_{cat}, sec^{-1}	K_m	k_{cat}, sec^{-1}
Laminarin	>30 g/liter	n.d.	pH 5.0, 50°C 1.03 ± 0.01 g/liter	142 ± 1	0.4 ± 0.05 g/liter	102 ± 3
β-Glucan	9 ± 4 g/liter	26 ± 4	5.5 ± 1.2 g/liter	125 ± 5	1.9 ± 0.1 g/liter	79 ± 1
Cellobiose	0.95 ± 0.02 mM	350 ± 5	pH 5.0, 40°C 1.20 ± 0.05 mM	89 ± 2	0.75 ± 0.02 mM	42 ± 1
*p*NPG	0.6 ± 0.01 mM	259 ± 2	0.44 ± 0.01 mM	160 ± 2	0.09 ± 0.01 mM	118 ± 4
K_i, mM*	2.73 ± 0.01		0.93 ± 0.03		0.51 ± 0.01	

Note: n.d., not determined.

* For inhibition of β-glucosidases by glucose.

gentiobiose and exhibited relatively low activities towards α-sophorose and laminaribiose.

Kinetic parameters of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei*. Kinetic parameters K_m and k_{cat} for the hydrolysis of *p*NPG, cellobiose, and the polysaccharide substrates β-glucan and laminarin are presented in Table 2. The highest value of k_{cat} was observed in the reaction of β-glucosidase of *A. japonicus* with cellobiose. The k_{cat} values of the two other β-glucosidases were 4-8-fold lower, the lowest k_{cat} value being exhibited by the β-glucosidase of *T. reesei*. The K_m values for cellobiose were approximately the same for the three enzymes. The turnover number of the β-glucosidase of *A. japonicus* in the reaction with *p*NPG exceeded 1.5-2-fold the corresponding parameters of the two other enzymes. The β-glucosidase of *T. reesei* exhibited the lowest K_m value for *p*NPG. In the reaction of hydrolysis of β-glucan and laminarin, the highest K_m value (and the lowest k_{cat} value) was observed in the case of β-glucosidase of *A. japonicus*, and the lowest K_m value was demonstrated for the β-glucosidase of *T. reesei*. The determined values of the kinetic parameters agree with the literature [22-24].

The extent of inhibition of β-glucosidases by glucose is an important characteristic of β-glucosidases, since the inhibition plays a negative role in enzymatic hydrolysis of cellulose in industrial production [23, 25]. Experiments on the inhibition by glucose were made using *p*NPG as the substrate (Table 2). It was shown that the isolated enzymes were competitively inhibited by the reaction product. The highest K_i value was shown for the β-glucosidase of *A. japonicus* (2.73 mM). Consequently, this enzyme is inhibited by glucose to lesser extent than other β-glucosidases, whose inhibition constants are 3-5-fold lower.

pH and temperature dependences of activities of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei*. The pH dependences of the β-glucosidases are similar (Fig. 1a). The enzymes exhibit maximal activity in acidic medium (optimal pH ranges were found to be 4.5-

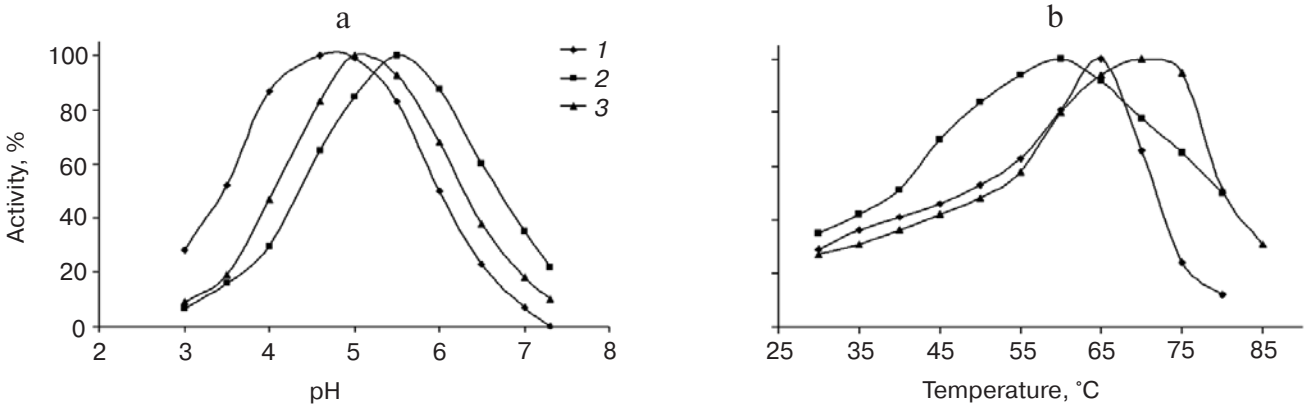

Fig. 1. Dependences of the activities of β-glucosidases of *A. japonicus* (*1*), *P. verruculosum* (*2*), and *T. reesei* (*3*) on pH (at 40°C) (a) and on temperature (at pH 5.0) (b) determined in the reaction of *p*NPG hydrolysis.

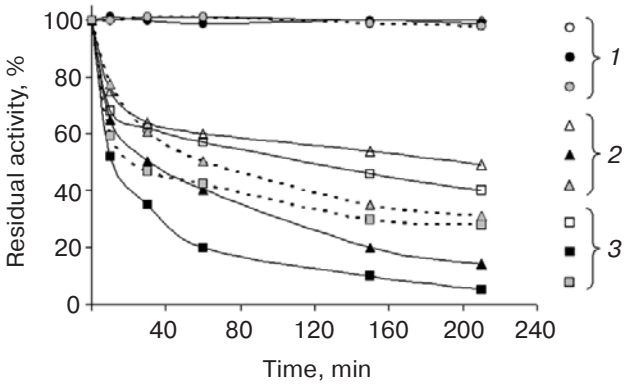

Fig. 2. Thermostability of β-glucosidases of *A. japonicus* (open symbols), *P. verruculosum* (closed symbols), and *T. reesei* (gray symbols). *1-3*) 30, 40, and 50°C, respectively. The activity was determined towards *p*NPG at 40°C and pH 5.0.

5.0, 5.0, and 5.5 for the β-glucosidases of *A. japonicus*, *T. reesei*, and *P. verruculosum*, respectively). The difference was that β-glucosidase of *A. japonicus* retained a detectible activity under more acidic conditions (relative to the pH optimum), while the β-glucosidase of *P. verruculosum* was active in the more alkaline region. Study of the influence of temperature on the enzymatic activity showed that the optimal temperature for the β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei* are 65, 60, and 70°C, respectively (Fig. 1b). It should be noted that the β-glucosidase of *P. verruculosum* exhibited more than 50% of its maximal activity in the range 40-80°C, while for the β-glucosidases of *A. japonicus* and *T. reesei* this temperature range was narrower – 48-72 and 50-80°C, respectively.

Thermostability of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei*. The thermostability of the glucosidases was studied at pH 5.0 (Fig. 2). All three β-glucosidases were stable at 30°C, retaining 90% of the original activity for more than 24 h. Increase in the temperature to 40°C resulted in significant inactivation of all three enzymes: after 3 h of incubation, the β-glucosidases of *A. japonicus* retained 50% of its activity, while the other enzymes lost 70-85% of their activity. At 50°C, the behavior of the enzymes did not change: the β-glucosidase of *P. verruculosum* was the least stable, losing 80% of its activity after 1 h of incubation, and the β-glucosidases of *T. reesei* and *A. japonicus* lost 57 and 43% of their activity, respectively, after the same time. Thus, the most stable was the β-glucosidase of *A. japonicus*, although the optimal temperature for its activity was not the highest among the investigated enzymes (to determine the optimal temperature, the enzymes were incubated at a certain temperature for 10 min).

Adsorption of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei* on MCC. It is known that some glycoside hydrolases contain a carbohydrate-binding module, for example the cellulose-binding module (CBM) [26]. To test whether the isolated β-glucosidases contain CBM, their adsorption to insoluble MCC was investigated. The β-glucosidase of *A. japonicus* showed virtually no adsorption to MCC (the ratio of the adsorbed protein constituted 6%), while in the case of the β-glucosidase of *P. verruculosum* 99% of the protein was adsorbed on MCC, which indicates the presence of the CBM in the structure of this enzyme. The β-glucosidase

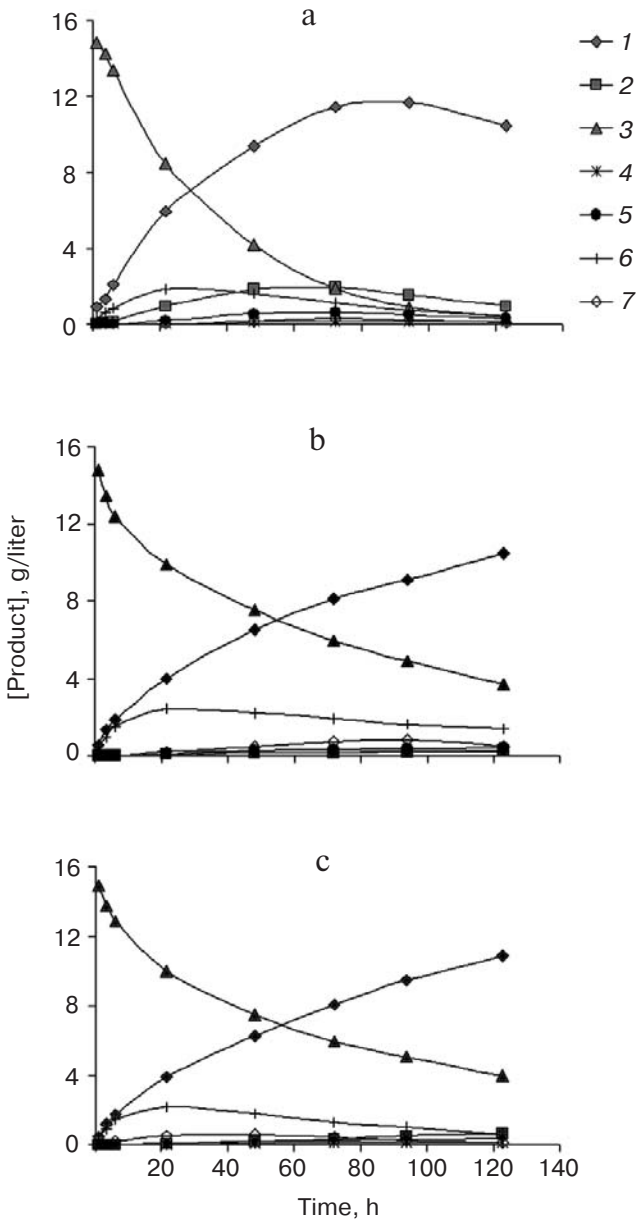

Fig. 3. Changes in the content of the transglucosidation products during the hydrolysis of cellobiose by β-glucosidases of *A. japonicus* (a), *P. verruculosum* (b), and *T. reesei* (c). *1*) Glucose; *2*) gentiobiose; *3*) cellobiose; *4*) α-sophorose; *5*) laminaribiose; *6*) trisaccharide; *7*) tetrasaccharide (30°C, pH 5.0, 50 mM cellobiose).

of *T. reesei* was adsorbed moderately (28%) and presumably did not contain the CBM.

Investigation of transglucosidation. The ability of the enzymes for transglucosidation decreases the concentration of the desired product of saccharification of cellulose (glucose), but at the same time this reaction can yield valuable products (different oligosaccharides) that are used as prebiotics.

At a high initial concentration of cellobiose (17.1 g/liter), a trisaccharide product of transglucosidation was accumulated at the beginning of the incubation with the isolated enzymes (Fig. 3). In this case, the cellobiose played the role of the nucleophile competing with a water molecule in breaking the enzyme–product complex. The formation of a tetrasaccharide with a simultaneous decrease in the trisaccharide content in the reaction mixture was also observed. In this case, the cellobiose was the substrate, and the trisaccharide formed in the first step served as the nucleophile. Maximal concentrations of the trisaccharide and tetrasaccharide were 1.8-2.4 and 0.4-0.8 g/liter, respectively. Maximal concentrations of these sugars (2.4 and 0.8 g/liter) were observed under the action on cellobiose of the β-glucosidase of *P. verruculosum*.

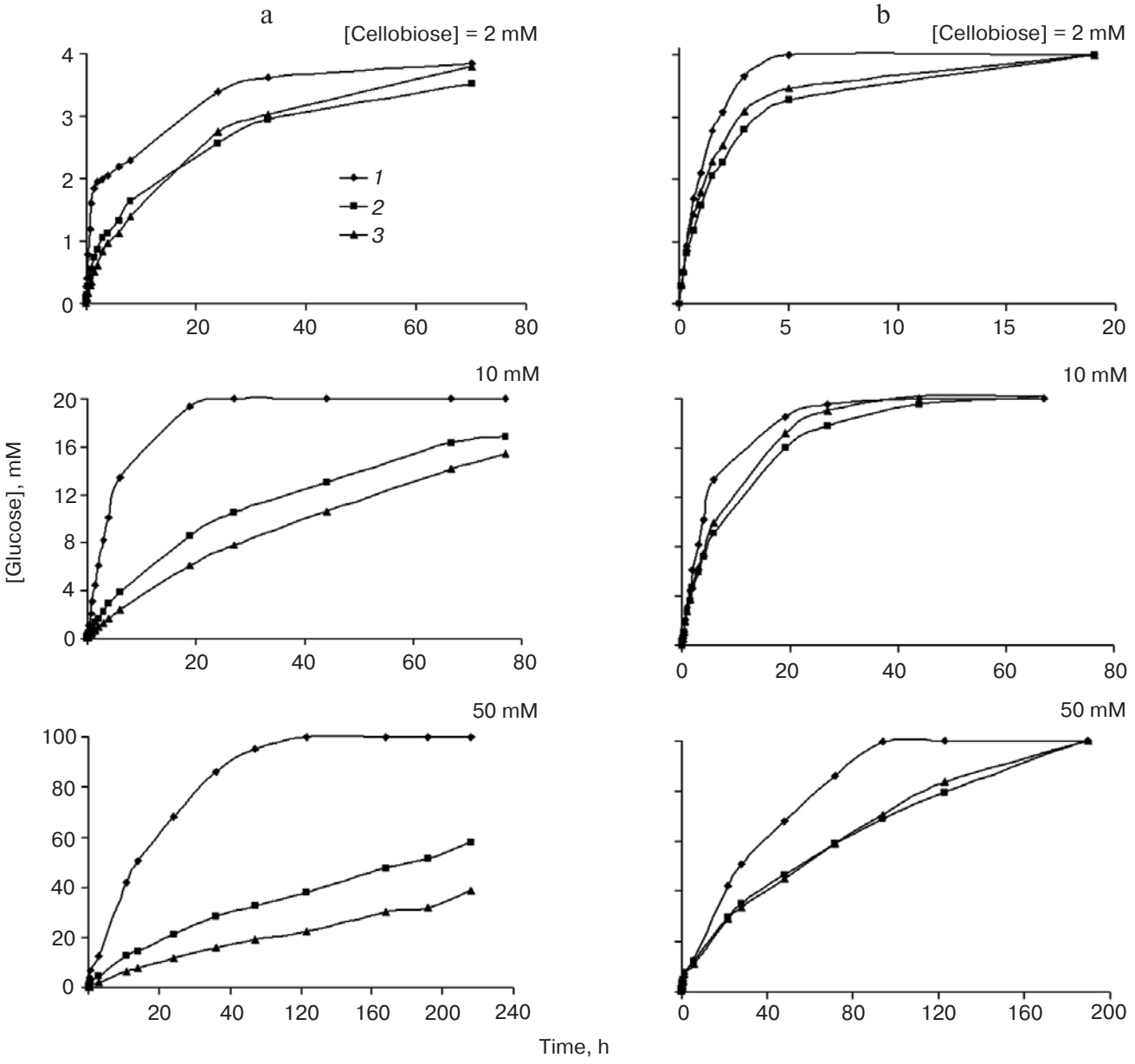

Fig. 4. Complete hydrolysis of cellobiose by β-glucosidases of *A. japonicus* (*1*), *P. verruculosum* (*2*), and *T. reesei* (*3*). The enzymes were normalized to concentration (0.3 mg/liter) (a) or to activity (0.025 U/ml) (b) at 30°C and pH 5.5.

A decrease in the original concentration of cellobiose and increase in the content of glucose during the hydrolysis resulted in the formation of gentiobiose, laminaribiose, and sophorose as the transglucosidation products (Fig. 3). In the presence of the β-glucosidase of *A. japonicus*, the concentration of gentiobiose was maximal (2.0 g/liter), concentration of laminaribiose was lower (0.5 g/liter), and the content of sophorose was the lowest (less than 0.1 g/liter). The β-glucosidase of *T. reesei* yielded only 0.8 g/liter of gentiobiose, 0.4 g/liter of laminaribiose, and 0.15 g/liter of sophorose. In the case of the β-glucosidase of *P. verruculosum*, the concentration of laminaribiose (0.4 g/liter) was higher than the concentration of gentiobiose (0.3 g/liter), and the content of sophorose (0.2 g/liter) was maximal among the three investigated β-glucosidases.

Complete hydrolysis of specific substrates. Kinetic characteristics and other properties of β-glucosidases influence the rate of cellobiose hydrolysis and glucose accumulation, as well as the extent of the hydrolysis of cellobiose. As mentioned above, important factors affecting the behavior of the β-glucosidases are concentrations of the substrate and the inhibitor (product). Hydrolysis of cellobiose was studied at different initial concentrations of cellobiose (0.68, 3.42, and 17.1 g/liter or 2, 10, and 50 mM, respectively). The enzymes were normalized to protein concentration (0.3 mg/ml) or to activity (0.025 U/ml).

When the enzymes were normalized to protein concentration, the shape of the kinetic curves was determined by the catalytic constants of the enzymes (Fig. 4a). The β-glucosidase of *A. japonicus* exhibited maximal rate of hydrolysis at all concentrations of cellobiose, since it has the highest k_{cat} value. The β-glucosidases of *P. verruculosum* and *T. reesei* have similar k_{cat} values, so their kinetic curves virtually coincided in the beginning of the hydrolysis. In the presence of 50 mM cellobiose, the β-glucosidase of *P. verruculosum* was more efficient: it hydrolyzed more substrate molecules in the same time since its K_i value is higher than that of the β-glucosidase of *T. reesei*.

When the enzymes were normalized to activity, the shape of the kinetic curves was determined by the inhibition constants of the enzymes (Fig. 4b). The β-glucosidase of *A. japonicus* exhibiting the highest K_i value demonstrated the highest rate and efficiency of hydrolysis at all substrate concentrations. The extent of the cellobiose conversion reached 100% in the presence of all three enzymes (in contrast to the case when the enzymes were normalized to protein concentration).

As mentioned above, the β-glucosidases differed in their substrate specificity (Table 1): the β-glucosidases of *P. verruculosum* and *T. reesei* exhibited high activity to laminarin and β-glucan (in contrast to the β-glucosidase of *A. japonicus*). In this connection, the polysaccharide substrates were completely hydrolyzed (the enzymes were normalized to protein concentration). The results of the hydrolysis of laminarin and β-glucan are presented in Fig. 5.

The β-glucosidases of *P. verruculosum* and *T. reesei* were more efficient in the hydrolysis of laminarin, since they exhibited the highest activity to this substrate: after 5 h of hydrolysis, the concentration of glucose was 2.5-fold higher than in the case of the β-glucosidase of *A. japonicus*. After 48 h of reaction, no increase in the concentration of the product was observed, and the reaction mixture was supplemented with a new portion of the enzymes of the same concentration that was in the beginning of the reaction. After the addition of the enzymes, the hydrolysis resumed in all cases. Therefore, the cessation of the process was connected with the inactivation of the enzymes. The extent of the conversion of laminarin after five days constituted 100% for the β-glucosidases of

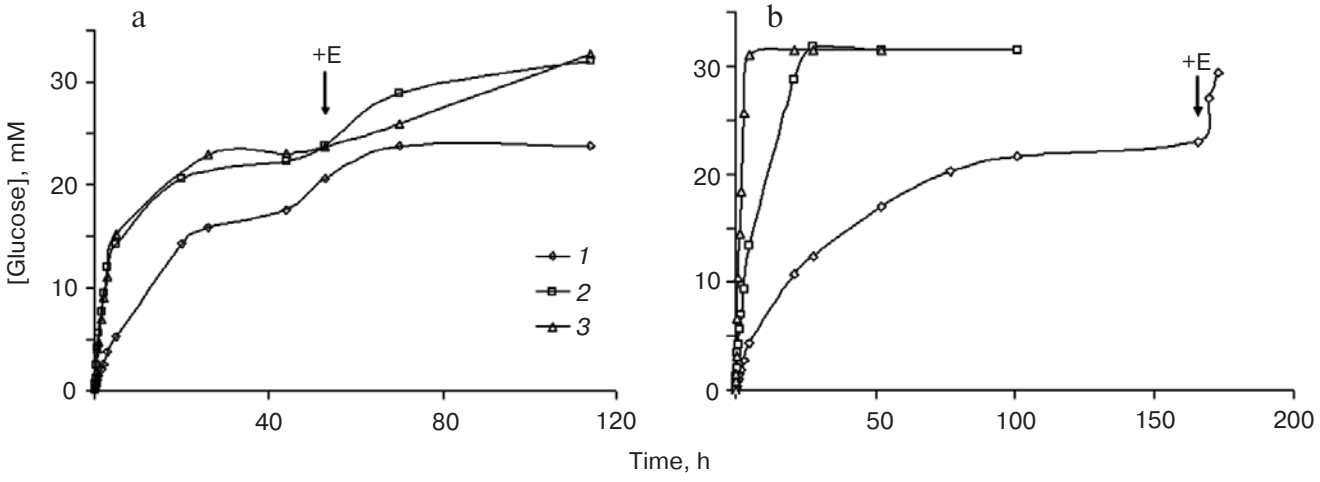

Fig. 5. Complete hydrolysis of laminarin (a) and β-glucan (b) by the β-glucosidases of *A. japonicus* (*1*), *P. verruculosum* (*2*), and *T. reesei* (*3*) (pH 5.0, 50°C). The addition of a new portion of the enzyme is designated as E.

P. verruculosum and *T. reesei* and 72% for the enzyme of *A. japonicus*.

Using β-glucan as the substrate, the difference in the extent of hydrolysis in the presence of different β-glucosidases was more pronounced (Fig. 5b). After 5 h of the reaction in the presence of the β-glucosidase of *T. reesei*, the extent of β-glucan conversion reached 100%, while for the β-glucosidases of *P. verruculosum* and *A. japonicus* this value constituted 42 and 12%, respectively. β-Glucan was hydrolyzed completely by the β-glucosidase of *P. verruculosum* after 24 h of incubation (without addition of new portions of the enzyme). For the enzyme of *A. japonicus*, the complete conversion of the substrate was observed after the addition of a new portion of the enzyme.

Thus, homogenous preparations of β-glucosidases of *A. japonicus*, *P. verruculosum*, and *T. reesei* were isolated, and their properties were investigated. The comparative study of the β-glucosidases allowed them to be divided into two groups in terms of their specificity. The β-glucosidase of *A. japonicus* exhibited high specific activity to the low molecular weight substrates cellobiose and *p*NPG (the activity towards cellobiose being higher than towards *p*NPG) and low activity to the polysaccharide substrates (β-glucan from barley and laminarin). The β-glucosidases of *P. verruculosum* and *T. reesei* exhibited relatively high activity to the polysaccharide substrates and lower activity to the low molecular weight substrates, the activity towards cellobiose being lower than the activity towards *p*NPG. Therefore, the β-glucosidase of *A. japonicus* can be characterized as an enzyme that is close to the "true" cellobiase, while the β-glucosidase of *P. verruculosum* and *T. reesei* are enzymes exhibiting cellobiase and exo-1,3/1,4-β-glucosidase activities.

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