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Isolation and Properties of Extracellular β-Xylosidases from Fungi *Aspergillus japonicus* and *Trichoderma reesei*

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Abstract—Homogeneous β-xylosidases with molecular mass values 120 and 80 kDa (as shown by SDS-PAGE), belonging to the third family of glycosyl hydrolases, were isolated by anion-exchange, hydrophobic, and gel-penetrating chromatography from enzyme preparations based on the fungi *Aspergillus japonicus* and *Trichoderma reesei*, respectively. The enzymes exhibit maximal activity in acidic media (pH 3.5-4.0), and temperature activity optimum was 70°C for the β-xylosidase of *A. japonicus* and 60°C for the β-xylosidase of *T. reesei*. Kinetic parameters of *p*-nitrophenyl β-xylopyranoside and xylooligosaccharide hydrolysis by the purified enzymes were determined, which showed that β-xylosidase of *A. japonicus* was more specific towards low molecular weight substrates, while β-xylosidase of *T. reesei* preferred high molecular weight substrates. The competitive type of inhibition by reaction product (xylose) was found for both enzymes. The interaction of the enzymes of different specificity upon hydrolysis of glucurono- and arabinoxylans was found. The β-xylosidases exhibit synergism with endoxylanase upon hydrolysis of glucuronoxylan as well as with α-L-arabinofuranosidase and endoxylanase upon hydrolysis of arabinoxylan. Addition of β-xylosidases increased efficiency of hydrolysis of plant raw materials with high hemicellulose content (maize cobs) by the enzymic preparation Celloviridine G20x depleted of its own β-xylosidase.

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Key words: xylanases, β-xylosidase, xylooligosaccharides, xylans, hydrolysis

A promising trend in biotechnology is plant mass bioconversion for obtaining biofuels (ethanol and butanol) as end products. A key stage of this process is the enzymic hydrolysis of the biomass to fermentable sugars. Because some microorganisms are able to convert pentoses (xylose and arabinose) to ethanol and butanol, plant xylans can also serve as an additional source of biofuels.

Xylan is the main hemicellulose component of the plant cell wall and the second most abundant natural polysaccharide after cellulose. The basis of its structure is a linear chain of xylopyranose residues joined to each other by β-1,4-bonds. Depending on xylan source, its main chain can contain different substituents – 4-O-methyl-D-glucuronic acid, D-glucuronic acid, and L-arabinofuranose being prevalent.

Xylan is extensively hydrolyzed by enzymes of the xylanase complex consisting of endoxylanases (1,4-β-xylan xylanohydrolase, EC 3.2.1.8), β-xylosidases (β-1,4-xylan xylohydrolase, EC 3.2.1.37), and arabinofuranosidases (α-L-arabinofuranoside arabinofuranosidase, EC 3.2.1.55) differing in their mechanism of action (endo- or exo-depolymerization). Owing to synergistic effect of their interaction, xylan hydrolysis by xylanase complexes is more rapid than that caused by the action of the individual enzymes. The end product of the β-xylosidase-catalyzed reaction is xylose.

The β-xylosidases isolated from fungi of *Aspergillus* (*A. niger*, *A. phoenicus*, *A. nidulans*, *A. carbonarius*), *Trichoderma*, *Talaromices*, and other genera have been characterized [1-7]. Recently information concerning properties of β-xylosidase from *A. japonicus* has appeared in the literature [8]. Note that *A. japonicus* is a promising xylanase producer: the amount of xylanases secreted by a mutant strain of this fungus is about 60% of its total extracellular enzymes [9].

The properties of purified β-xylosidases from *A. japonicus* and *T. reesei*, their abilities to hydrolyze polymer substrates and saccharify natural xylan-containing substrates, as well their synergistic effect upon interaction of the xylanase complex enzymes have been studied in this work.

Abbreviations: RS, reducing sugar.

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MATERIALS AND METHODS

Enzymes. Laboratory enzyme preparations of *A. japonicus* and *T. reesei* obtained in the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences were used in this work. These preparations were lyophilized ultrafiltrates of fungal culture liquid. The commercial preparation Celloviridine G20x (Biovet, Bulgaria) was also used. Commercial preparation Xybeten-Xyl (Biovet) and laboratory enzyme preparation of *Penicillium canescens* obtained from the Institute of Biochemistry and Physiology of Microorganisms were used for isolation of homogeneous endoxylanase II of *T. reesei* and α-L-arabinofuranosidase of *P. canescens*.

Substrates. Glucuronoxylan from birch, xyloglucan, *p*-nitrophenyl β-D-xylopyranoside, and *p*-nitrophenyl α-D-arabinofuranoside from Sigma (USA), arabinoxylan from wheat, xylobiose, xylotriose, and xylotetraose from Megazyme (Australia), xylose from Merck (Germany), and maize cobs from Dyadic, Inc. (USA) were used in this work.

Determination of enzyme activities. Enzyme activities towards polysaccharide substrates were determined according to the method of Somogyi–Nelson by the initial rate of reducing sugar (RS) formation [10]. Activities towards xylooligosaccharides were determined by the initial rate of RS formation using the bicinchoninate technique [11]. Enzyme activities toward low molecular weight synthetic substrates (*p*-nitrophenyl derivatives of sugars) were determined by the initial rate of *p*-nitrophenol formation [10].

In all cases, the enzyme amount that catalyzed formation of 1 μmol product in 1 min under certain conditions was taken as one activity unit.

Protein content in samples was determined by absorption at 280 nm or by the Lowry method with BSA as standard.

The yield of sugars during enzymatic hydrolysis of natural substrates was determined according to the Somogyi–Nelson technique [10].

Isolation of homogeneous β-xylosidases of *A. japonicus* and *T. reesei*. An FPLC chromatographic system, columns, and carriers of Pharmacia (Sweden) were used for enzyme isolation. The low-pressure Econo-System (Bio-Rad, USA) was used for sample preparation and for their desalting and buffer exchange.

The enzyme preparation was desalted on an Acrylex P2 column (Reanal, Hungary) in 20 mM piperazine-HCl buffer, pH 5.5 (for the *A. japonicus* preparation), or in 20 mM imidazole-HCl, pH 7.5 (for the *T. reesei* preparation). This was followed by anion-exchange chromatography on a Source 15Q column. The sample was applied in initial buffer at pH 5.5 (for *A. japonicus*) or pH 7.5 (for *T. reesei*); bound proteins were eluted in a NaCl concentration gradient.

The fraction containing β-xylosidase from *A. japonicus* was separated by chromatography on a column with the hydrophobic carrier Phenyl Superose. Dry ammonium sulfate was added to the fraction with stirring to 2.5 M. Then the sample was applied onto the column equilibrated with 50 mM Na-acetate buffer, pH 5.0, containing 2.5 M ammonium sulfate. Bound proteins were eluted in a reverse ammonium sulfate concentration gradient of 2.5-0 M. The purified protein was separated from ammonium sulfate on a column of Sephadex G25 in 50 mM Na-acetate buffer, pH 5.0.

In the case of β-xylosidase of *T. reesei*, the multienzyme fraction obtained after anion-exchange chromatography and containing β-xylosidase activity underwent gel filtration on a Superose 12 column in 50 mM Na-acetate buffer, pH 5.0, 0.1 M NaCl.

Homogeneous 21 kDa endoxylanase II of *T. reesei* and 70 kDa α-L-arabinofuranosidase of *P. canescens* belonging to families 11 and 51 of glycosyl hydrolases, respectively, were isolated according to previously described methods [12, 13].

Determination of molecular characteristics of purified enzymes. Protein electrophoresis was carried out in 12% SDS-polyacrylamide gel on a Mini Protein device (Bio-Rad) according to the manual. Protein bands were stained with Coomassie G-250.

Determination of low molecular weight products of natural substrate hydrolysis. Substrate hydrolysis products were analyzed using refractivity detection on a Diasorb-130-Amine column with bound aminopherase (BioKhimMak, Russia). An acetonitrile mixture with water (75 : 25 v/v) was used as the eluent, the flow rate being 1 ml/min. Acetonitrile (90 μl) was added to 30 μl boiled sample taken during hydrolysis and the mixture was centrifuged for 5 min at 14,000*g*. Xylooligosaccharides with polymerization extent 1-4, glucose, and arabinose were used as standards.

RESULTS AND DISCUSSION

Isolation of β-xylosidases. The schemes for purification of the individual enzymes included a number of successive chromatographic stages. The initial multienzyme preparations were fractionated on Source 15Q.

It should be noted that during the separation of the *A. japonicus* preparation, maximal activity towards *p*-nitrophenyl β-xylopyranoside was detected in two fractions eluting at 0.15 and 0.18 M NaCl (Fig. 1a). It became apparent during further purification and determination of properties that the two enzymes corresponding to these fractions are isoforms of the same β-xylosidase because they have similar properties: similar molecular mass and specific activity towards *p*-nitrophenyl β-xylopyranoside. This was confirmed by mass-spectrometric analysis: nine identical peptides (m/z) were found in spectra that pro-

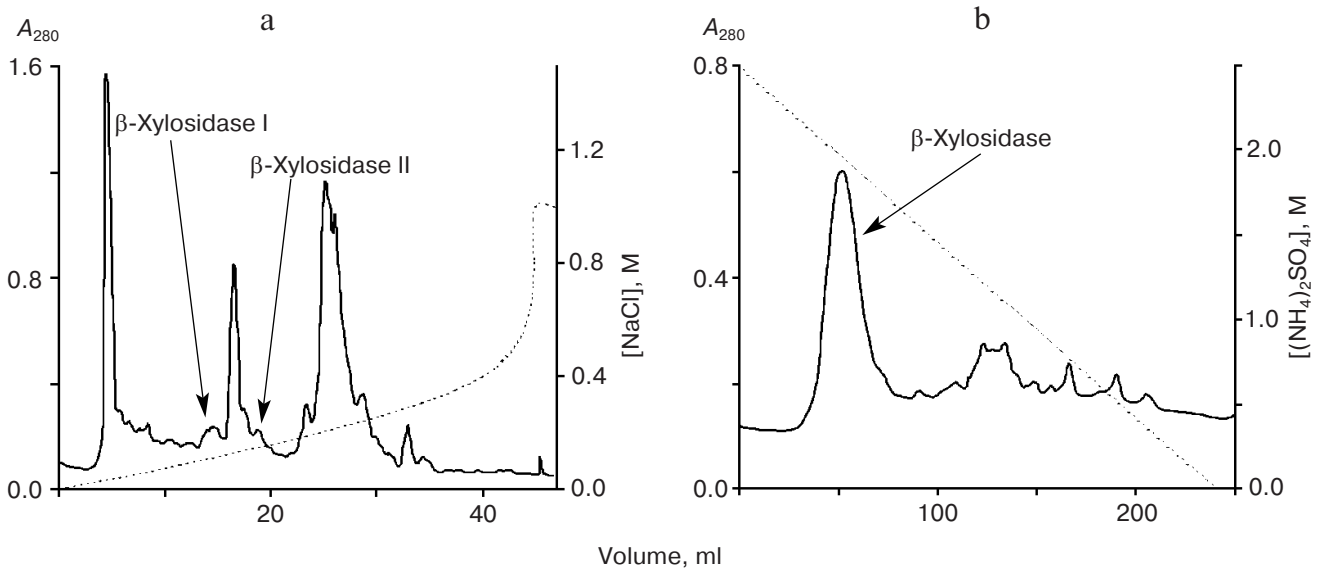

Fig. 1. Isolation of homogeneous β-xylosidase of *A. japonicus* using anion-exchange chromatography on Source 15Q at pH 5.5 (a) with subsequent hydrophobic chromatography on Phenyl Superose (b). The dashed line shows the salt gradient concentration.

vided for overlapping of 23% of the amino acid sequence by highly homologous β-xylosidase of *A. niger*. The fact that the isoforms eluted at different NaCl concentrations is might be explained by different posttranslational modifications of the two protein peaks. All subsequent investigations were carried out with only the first eluted isoform, while the second is not mentioned further in this work.

In the next stage of purification of the *A. japonicus* β-xylosidase (Fig. 1b) fractions collected after anion-exchange chromatography and containing β-xylosidase activity were separated by chromatography on a column with the hydrophobic carrier Phenyl Superose in an inverse gradient of ammonium sulfate (from 2.5 to 0 M). In this case the fraction exhibiting activity towards *p*-nitrophenyl β-xylopyranoside was eluted at 2.1-1.8 M $(NH_4)_2SO_4$. This stage of separation removed minor admixtures.

During purification of β-xylosidase of *T. reesei*, at the first stage (anion-exchange chromatography) maximal activity towards *p*-nitrophenyl β-xylopyranoside corresponded to the fraction eluted at 0.13 M NaCl (Fig. 2a).

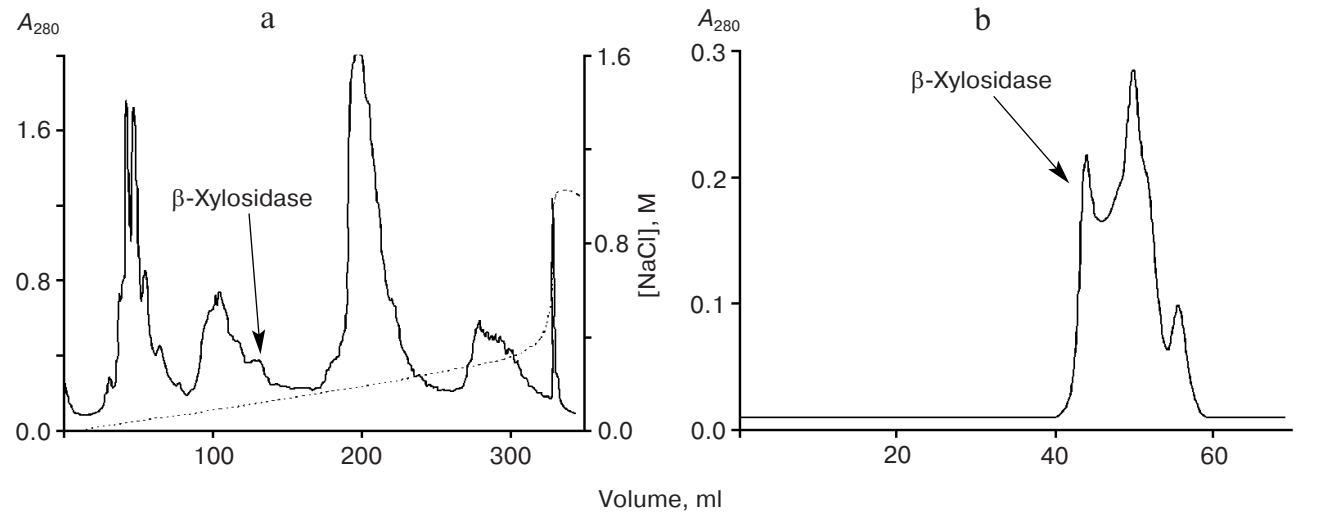

Fig. 2. Isolation of homogeneous β-xylosidase of *T. reesei* using anion-exchange chromatography on Source 15Q at pH 7.5 (a) with subsequent gel-penetrating chromatography on Superose 12 (b). The dashed line shows the salt gradient concentration.

The β-xylosidase was finally purified by gel filtration on a column of Superose 12 (Fig. 2b). The fraction corresponding to the first of three chromatographic peaks contained the *T. reesei* β-xylosidase.

Properties of purified β-xylosidases. As shown in the literature [1-8], fungal β-xylosidases can significantly differ in their biochemical and catalytic properties. Enzymes with molecular mass values from 26 to 420 kDa are described in the literature (molecular mass above 120 kDa usually corresponds to dimeric and tetrameric forms). A few known β-xylosidases are classified on the basis of analysis of hydrophobic clusters and comparison of amino acid sequence. According to the classification they are included in four glycosyl hydrolase families: 3, 39, 43, and 52. All known fungal β-xylosidases belong to family 3.

In this work, purification produced homogeneous β-xylosidases from *A. japonicus* and *T. reesei*; according to SDS-PAGE, their molecular mass values were 120 and 80 kDa, respectively (Fig. 3).

MALDI-TOF mass spectrometry of the purified protein tryptic hydrolysates showed that both enzymes belong to glycoside hydrolase family 3 (data not shown).

The pH and temperature dependence of the enzyme activities and enzyme stability at different temperatures were studied. Maximal enzyme activity was registered at pH 4.0 and 3.5 for β-xylosidases from *A. japonicus* and *T. reesei*, respectively. These values correlate well with data from the literature: optimal pH range is 4.0-4.5 for xylosidases isolated from fungi of *Aspergillus* genus and 3.0-4.5 for enzymes from fungi of genus *Trichoderma* [2-8].

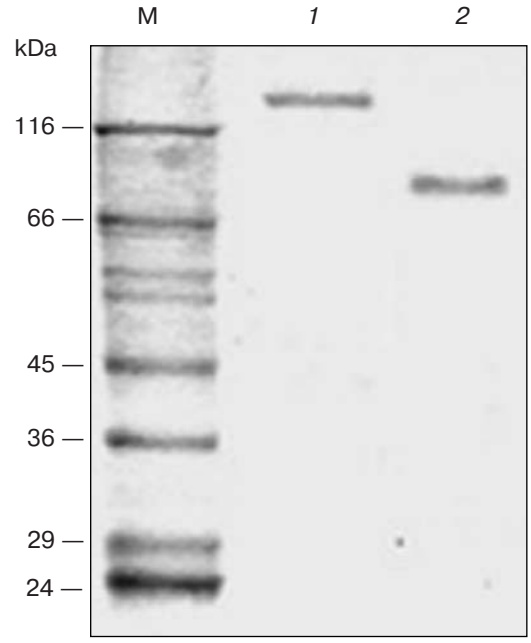

Fig. 3. SDS-PAGE of purified β-xylosidases from *A. japonicus* (*1*) and *T. reesei* (*2*). M, protein markers (molecular masses in kDa are shown).

Table 1. Specific activities of homogeneous β-xylosidases towards different substrates

Substrate	Specific activity, units/mg	
	A. japonicus	*T. reesei*
	pH 5.0, 40°C	
p-Nitrophenyl β-xylopyranoside	42.6	16.0
p-Nitrophenyl α-arabinofuranoside	3.9	0.1
Xylobiose	28.4	25.0
	pH 5.0, 50°C	
Glucuronoxylan	7.0	14.2
Arabinoxylan	0.1	0.9
Xyloglucan	0	0

Studying the temperature effect on activity and stability of β-xylosidases showed that the enzyme of *A. japonicus* is more thermostable than that of *T. reesei*: temperature optimum of activity of β-xylosidase *of A. japonicus* was 70°C and that of *T. reesei* was 60°C. Investigation of enzyme stability showed that both enzymes completely retained initial activity for 3 h at 40 and 50°C. Increasing temperature to 60°C did not change activity of the *A. japonicus* enzyme after incubation for 3 h, but noticeable inactivation of the *T. reesei* enzyme was observed (only 52% of the initial activity remained after incubation for 3 h). Increasing temperature to 70°C resulted in complete inactivation of β-xylosidase of *T. reesei* in 20 min, whereas the activity of the β-xylosidase of *A. japonicus* decreased by only one third (63% activity remained after 3 h).

Specific activities of the purified β-xylosidases towards different substrates were determined (Table 1). The *A. japonicus* β-xylosidase exhibited 2.5-fold higher activity towards low molecular weight synthetic substrate *p*-nitrophenyl β-xylopyranoside compared to the *T. reesei* β-xylosidase.

Both of the enzymes exhibited activity towards *p*-nitrophenyl α-D-arabinofuranoside, but it was 10-15 times lower than that towards *p*-nitrophenyl β-xylopyranoside. β-Xylosidases exhibiting activity towards other *p*-nitrophenyl derivatives appear in the literature extremely rarely. β-Xylosidase of *A. carbonarius* exhibiting activity towards *p*-nitrophenyl α-arabinofuranoside was described [3]. Activity of β-xylosidase of *A. japonicus* towards *p*-nitrophenyl derivatives of β-glucopyranoside and α-arabinofuranoside was, respectively, 31.6 and

Table 2. Kinetic parameters of *p*-nitrophenyl β-xylopyranoside and xylooligosaccharide hydrolysis by β-xylosidases (40°C, pH 5.0)

Substrate	K_m, mM	V_{max}/[E]*, sec^{-1}	K_i, mM	K_m, mM	V_{max}/[E]*, sec^{-1}	K_i, mM
	A. japonicus			*T. reesei*		
p-Nitrophenyl β-xylopyranoside	0.33 ± 0.04	98 ± 3	2.9 ± 0.2	0.51 ± 0.04	12 ± 1	1.4 ± 0.1
Xylobiose	4.6 ± 1.8	66 ± 13	–	7.2 ± 4.1	56 ± 13	–
Xylotriose	5.7 ± 1.9	43 ± 8	–	6.4 ± 2.6	70 ± 10	–
Xylotetraose	4.4 ± 2.2	29 ± 8	–	5.3 ± 2.2	85 ± 12	–

* Values are calculated for theoretical molecular masses 87.7 and 87.6 kDa of β-xylosidase from *A. japonicus* and *T. reesei*, respectively.

17.7% of its activity towards *p*-nitrophenyl β-xylopyranoside [8].

The specific activities of the two β-xylosidases towards xylobiose were similar to each other. Both enzymes were active towards polymeric substrate glucuronoxylan (activity of β-xylosidase of *T. reesei* was twice as high). Activity of both β-xylosidases towards arabinoxylan was significantly lower than that towards glucuronoxylan. Neither enzyme exhibited activity towards xyloglucan, i.e. they were not able to cleave xylose present in side chains attached to the main chain of this substrate.

Kinetic parameters of specific substrate hydrolysis. All known β-xylosidases are active towards synthetic *p*-nitrophenyl β-xylopyranoside, which has become a model substrate for studying properties of these enzymes. The K_m values for this substrate are high, of the order of 0.2-2.4 mM [2-8]. The K_m values obtained for β-xylosidases of *A. japonicus* and *T. reesei*—0.33 and 0.51 mM, respectively—fall within this range (Table 2).

β-Xylosidases differ by affinity to short and long oligosaccharides depending on which they are assigned either to β-xylosidases or to exo-β-xylanases. Thus, β-xylosidase of *A. nidulans* [4] exhibited higher specificity towards xylobiose, whereas xylooligosaccharides of higher molecular weight were more preferable for the enzymes of *A. phoenicus* [2], *A. japonicus* [8], and *T. reesei* [6].

Correlation of kinetic parameters of xylooligosaccharide hydrolysis by the enzymes showed that in the case of the *A. japonicus* β-xylosidase the V_{max}/[E] value is the highest for xylobiose, and in the case of the *T. reesei* β-xylosidase it is the highest for xylotetraose (Table 2). Therefore, the *A. japonicus* β-xylosidase is an enzyme specific to low molecular weight substrates, whereas the *T. reesei* β-xylosidase is an exo-β-xylanase specific to high molecular weight substrates. For both β-xylosidases the K_m value was practically independent of the substrate polymerization degree.

When *p*-nitrophenyl β-xylopyranoside was used as the substrate, the inhibition of the enzyme by xylose was studied and competitive type of inhibition was determined for both β-xylosidases (data not shown). Calculated inhibition constants are shown in Table 2. The *A. japonicus* β-xylosidase has a higher K_i value; the enzyme is inhibited by xylose to a lesser extent than the *T. reesei* β-xylosidase.

Hydrolysis of polymeric substrates. Synergistic action of xylanase enzyme complexes. Natural xylans are structurally complex polysaccharides with numerous substituents of the main chain (or short side chains). Therefore, extensive hydrolysis requires the use of a complex of enzymes having different specificities.

Glucuronoxylan and arabinoxylan were hydrolyzed by individual enzymes (the studied β-xylosidases, endoxylanase II of *T. reesei*, and α-L-arabinofuranosidase of *P. canescens*) and by different combinations of these enzymes. Figure 4 shows results of hydrolysis by the *A. japonicus* β-xylosidase (similar results were obtained for the *T. reesei* enzyme). The figure also shows theoretical curves calculated using data of the summarized action of the individual enzymes.

The difference between the studied β-xylosidases was a noticeable prevalence of the *T. reesei* β-xylosidase over the *A. japonicus* β-xylosidase during the initial part (3-4 days) of glucuronoxylan hydrolysis by the individual β-xylosidases (extent of conversion 25 and 6%, respectively), which again confirms the higher affinity of the first enzyme to high molecular weight substrate and of the second enzyme to low molecular weight substrate. A *T. reesei* β-xylosidase that is highly active towards unbranched xylan (xylan of wheat straw and beech tree) is described in the literature [6].

Synergistic effect upon combined action of β-xylosidase and endoxylanase was clearly shown in glucuronoxylan hydrolysis (Fig. 4a): the experimental curve of RS accumulation in response to the enzyme mixture is above the theoretical value. In this case a mixture of the *A. japonicus* β-xylosidase with endoxylanase (which quickly hydrolyzes glucuronoxylan to xylooligosaccharides) was

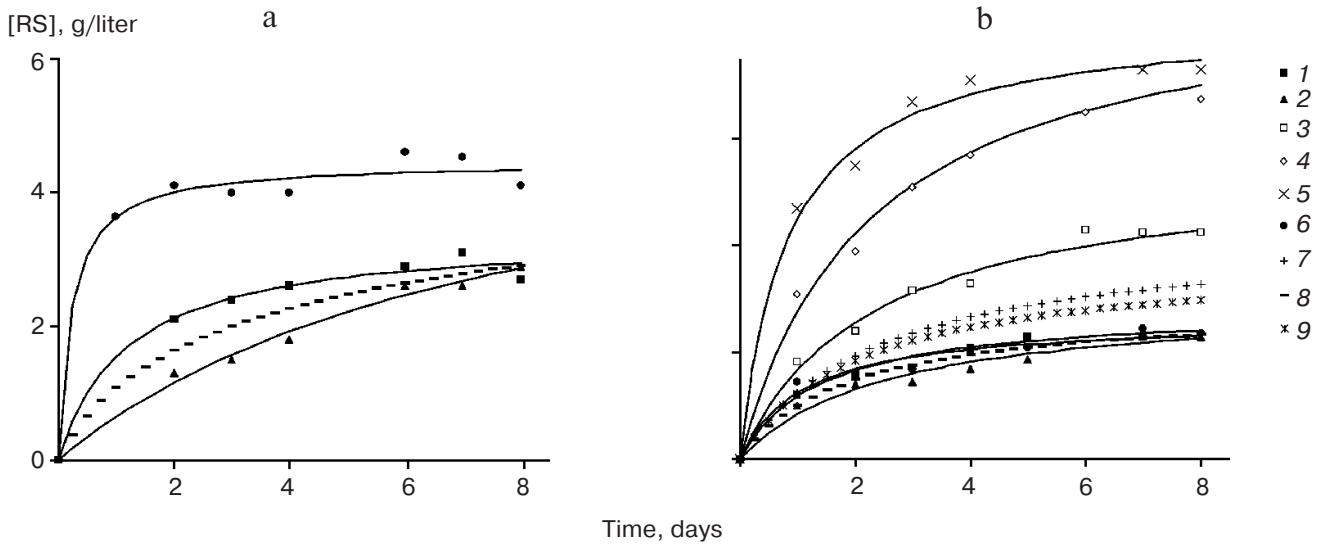

Fig. 4. Glucuronoxylan (a) and arabinoxylan (b) hydrolysis by the *A. japonicus* β-xylosidase alone and its mixtures with *T. reesei* endoxylanase II and *P. canescens* α-L-arabinofuranosidase. Hydrolysis conditions: substrate concentration 10 g/liter, pH 5.0, 40°C. Designations of experimental data: *1*) endoxylanase; *2*) β-xylosidase; *3*) arabinofuranosidase; *4*) β-xylosidase mixture with arabinofuranosidase; *5*) ternary mixture of β-xylosidase, endoxylanase, and arabinofuranosidase; *6*) β-xylosidase mixture with endoxylanase. Designations of theoretical curves calculated on the basis of data on summarized action of individual enzymes are as follows: *7*) β-xylosidase mixture with arabinofuranosidase; *8*) β-xylosidase mixture with endoxylanase; *9*) β-xylosidase mixture with endoxylanase and arabinofuranosidase.

more efficient than a mixture of the *T. reesei* β-xylosidase with endoxylanase, and already during the first day of hydrolysis it provided for a high extent (39%) of substrate conversion (for the *T. reesei* β-xylosidase mixture with endoxylanase the extent of substrate conversion in the same time was 23%). The cessation of the process catalyzed by the β-xylosidase–endoxylanase mixtures at the level of 45% conversion is evidently explained by steric hindrances due to the presence of glucuronic acid residues.

Analysis by HPLC of the composition of the glucuronoxylan hydrolysis products showed that the individual endoxylanase forms xylose, xylobiose (main product), and xylotriose as well as of higher molecular weight oligosaccharides. Xylose was the main product of the individual β-xylosidases. The xylose yield after the action of β-xylosidase–endoxylanase mixtures increased due to hydrolysis by β-xylosidase of xylobiose, xylotriose, and other oligosaccharides produced by endoxylanase (data not shown).

Synergism in action of different enzymes was also observed in arabinoxylan hydrolysis (Fig. 4b). The individual β-xylosidases relatively weakly hydrolyzed the substrate (conversion extent in eight days was 9 and 21%, respectively, for β-xylosidases of *A. japonicus* and *T. reesei*), because the presence of side substituents in the arabinoxylan main chain hinders the functioning of the β-xylosidases. The β-xylosidase complex with endoxylanase was also of relatively low efficiency, the extent of exhaustive hydrolysis being 21-22%. The ternary enzyme complex (β-xylosidase + endoxylanase + α-L-arabinofuranosidase) was most efficient: the arabinoxylan conversion extent was 68-71%. In this case, despite the difference in the individual β-xylosidase activities towards the substrate (Table 1), this difference was the same as for the ternary enzyme complex and the final extent of hydrolysis was practically identical. Note that the β-xylosidase

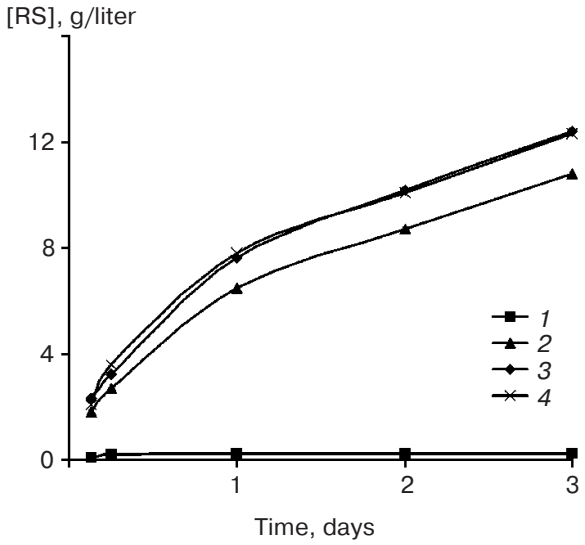

Fig. 5. Hydrolysis of maize cobs by Celloviridine G20x preparation alone (*2*) and its mixtures with the β-xylosidase of *A. japonicus* (*3*) or *T. reesei* (*4*). Control (*1*), no enzyme was added. Hydrolysis conditions: substrate concentration 50 g/liter, Celloviridine G20x concentration 2 mg/g, and concentration of the β-xylosidases was 0.2 mg/g of substrate dry weight, 50°C, pH 5.0.

and α-L-arabinofuranosidase were also capable of efficient hydrolysis of arabinoxylan in the absence of endoxylanase, the conversion extent being 57-64%.

Xylose was the main product among those of arabinoxylan hydrolysis by the individual β-xylosidases, a small amount of arabinose also being observed after hydrolysis by the *T. reesei* β-xylosidase. The same composition of products (xylose and arabinose) was obtained using different enzyme combinations (β-xylosidase + α-L-arabinofuranosidase or β-xylosidase + endoxylanase + α-L-arabinofuranosidase). No accumulations of xylooligosaccharides were observed because they were completely hydrolyzed to xylose and arabinose.

Efficiency of β-xylosidase during saccharification of natural raw materials (maize cobs). To estimate the contribution of β-xylosidase to the efficiency of plant raw material hydrolysis and comparison of their hydrolytic abilities, maize cobs treated in advance by vapor explosion were hydrolyzed by a commercial enzyme preparation (Celloviridine G20x) and its mixtures with β-xylosidases (Fig. 5). Addition of β-xylosidases to Celloviridine G20x exhibiting low β-xylosidase activity increased efficiency of hydrolysis. In this case no difference was observed between the efficiencies of the xylosidases of *A. japonicus* and *T. reesei*.

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