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Isolation and Properties of Xyloglucanases of *Penicillium* sp.

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Abstract—Using chromatographic technique, xyloglucanase (XG) A (25 kDa, p*I* 3.5, 12th glycosyl hydrolase family) was isolated from the enzyme complex secreted by the mycelial fungus *Penicillium canescens*, and xyloglucanases XG 25 (25 kDa, p*I* 4.1, 12th glycosyl hydrolase family) and XG 70 (70 kDa, p*I* 3.5, 74th glycosyl hydrolase family) were isolated from the enzyme complex of *Penicillium verruculosum*. Properties of the isolated enzymes (substrate specificity, optimal ranges of pH and temperature for enzyme activity and stability, effect of metal ions on catalytic activity) were compared with the properties of xyloglucanases XG 32 of *Aspergillus japonicus*, XG 78 of *Chrysosporium lucknowense*, and XG of *Trichoderma reesei*. The gene *xegA* encoding XG A of *P. canescens* was isolated, and the amino acid sequence of the corresponding protein was determined.

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Key words: xyloglucanase, xyloglucan, *Penicillium canescens*, *Penicillium verruculosum*

Xyloglucan is a widespread hemicellulose polysaccharide of plant cell walls. It contains mainly D-glucose and D-xylose in the ratio of approximately 4 : 3, less amount of D-galactose, and also can contain L-fucose and L-arabinose. The backbone of the polymeric molecule of xyloglucan is composed of β-1,4-bound D-glucopyranoside residues, most of which is substituted at the C6-carbon atom by single residues of D-xylopyranose attached by α-1,6-bonds. The xyloside groups, in turn, can be substituted by residues of D-galactopyranose attached by β-1,2-bonds and L-fucopyranose attached by α-1,2-bonds. The side groups are distributed orderly: three substituents (one after another) are followed by a space [1]. According to the simplified nomenclature suggested by Fry et al. [2], non-substituted glucose residues of the xyloglucan are designated as G, glucose residues containing a xylosyl substituent are designated as X, the residues with xylosyl-galactose substituents as L, and the segment containing fucose as F. Thus, the main structural motif of xyloglucan Xyl_3Glc_4 is designated as XXXG. Xyloglucan from the seeds of the tamarind *Tamarindus indica* that is now the most studied [3, 4] exhibits the following pattern of disposition of the galactosyl substituents: the xylose residue that is distant from the reducing end of the structural element XXXG never contains galactosyl substituent, while two other xylose residues may contain non-randomly distributed substituents [3, 4]. Thus, the structural element of tamarind xyloglucan can be represented as X(X,L)(X,L)G.

The backbone of xyloglucan can be cleaved by many endo-β-1,4-glucanases by the endo-depolymerase mechanism. Besides endoglucanases, whose substrates are mainly cellulose and different β-glucans, there are xyloglucanases (XG) that are highly specific towards xyloglucan, being virtually inert towards the non-substituted β-glucans and carboxymethyl cellulose (CMC). The xyloglucanase of fungal origin (XEG of *Aspergillus aculeatus*, 34 kDa, p*I* 3.4, 12th family of glycosyl-hydrolases) was first described in 1999 [5]. The authors named this enzyme as xyloglucan-specific endo-β-1,4-glu-

Abbreviations: CMC, carboxymethyl cellulose; MMD, molecular-mass distribution; XG, xyloglucanase.

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canase. Since then, the number of works devoted to enzymes involved in the transformation of xyloglucan has been increasing each year. A growing interest in xyloglucanases is connected with the possibility of their application in a number of important biotechnological processes, such as conversion of the plant waste, modification of xyloglucans to endow them required rheological properties in food and feed industries, in pulp and paper industry, treatment of the fabrics to change its brightness and color and to remove the fuzz from the surface of textile materials in the textile industry, etc.

The goal of the present work was isolation of xyloglucanases of *Penicillium canescens* and *P. verruculosum* and investigation of their properties in comparison with other xyloglucanases of fungal origin (XG 32 of *Aspergillus japonicus*, XG 78 of *Chrysosporium lucknowense*, and XG of *Trichoderma reesei* [6]).

MATERIALS AND METHODS

Enzyme preparations. The following dry enzyme preparations were used: PCA10 obtained using the fungal strain *P. canescens* (xyloglucanase activity determined with tamarind seed xyloglucan constituted 1500 U/g) and B221-151 obtained using the fungal strain *P. verruculosum* (xyloglucanase activity towards tamarind seeds xyloglucan constituted 5000 U/g). Both preparations were obtained from the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Science (Pushchino, Moscow Region).

Substrates. To determine enzymatic activities, the following substrates were used: barley β-glucan (medium viscosity) and xyloglucan from tamarind seeds (Xyl/Glc/Gal/Ara = 35 : 45 : 16 : 4) (Megazyme, Australia); sodium salt of CMC (medium viscosity, Sigma, USA).

Isolation and purification of xyloglucanases. Xyloglucanases from the *P. canescens* PCA10 and *P. verruculosum* B221-151 preparations were isolated in three steps: preliminary purification of the preparation, ion-exchange chromatography of the complex, and gel permeation (for PCA10) or hydrophobic (for B221-151) chromatography of the xyloglucanase fraction.

The preparations were precipitated with ammonium sulfate (80% saturation at 25°C). The precipitates were dissolved in 0.1 M sodium-acetate buffer, pH 5.0, and desalted on an Acrylex P-2 column (Reanal, Hungary) using an Econo-System liquid chromatograph (Bio-Rad, USA). The proteins were eluted with 0.02 M bis-Tris-HCl (pH 6.0 in the case of PCA10 and pH 6.2 in the case of B221-151) at flow rate of 1 ml/min. Subsequent purification was performed using an FPLC system (Pharmacia, Sweden). Ion-exchange chromatography was performed using a Source 15Q HR 16/5 column (Pharmacia). A sample containing 100 mg protein was applied on the column equilibrated with 0.02 M bis-Tris-HCl (pH 6.0 for PCA10 and 6.2 for B221-151). The bound proteins were eluted with 300 ml of a linearly increasing NaCl gradient at 5 ml/min. When necessary (in the case of B221-151), the buffer in the pooled fractions was changed using a Biogel P4 column (Bio-Rad).

PCA10 fractions exhibiting xyloglucanase activity were purified by gel permeation chromatography using a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 0.1 M Na-acetate buffer, pH 5.0. Maximal volume of the applied fraction was 200 μl, and the flow rate was 0.3 ml/min.

B221-151 fractions exhibiting xyloglucanase activity were purified by hydrophobic chromatography using a Source 15 Isopropyl column (Pharmacia) equilibrated with 1.7 M $(NH_4)_2SO_4$ in 0.05 M Na-acetate buffer, pH 5.0. The bound protein was eluted with 240 ml of a linearly decreasing ammonium sulfate gradient at flow rate of 2 ml/min. The resulting fractions were desalted on a Sephadex G-25 column (Pharmacia) in 0.1 M sodium-acetate, pH 5.0, at 0.5 ml/min.

Protein content in the samples was determined by the Lowry method using BSA as the standard [7] or by absorption at 280 nm.

Searching for proteins that are homologous to the isolated xylanases [8-12]. After SDS-PAGE of protein samples, the areas of the gel corresponding to the protein bands of xyloglucanases were treated with trypsin [9] (genetically modified trypsin for sequencing (Promega, USA), 5 ng/ml in 0.05 M NH_4HCO_3). The peptides were extracted with 20% acetonitrile containing 0.1% trifluoroacetic acid. The protein hydrolysate was analyzed by MALDI-TOF mass spectrometry using a Reflex III mass spectrometer (Bruker Daltonics, Germany) in the Department of Proteomic Investigation of the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences. Based on the results, the peptides with identical molecular masses among the known glycosyl-hydrolases were searched using the MASCOT program (http://matrixscience.com/) and NCBI, MSDB, and SWISS-PROT protein databases.

Amino acid sequences of the peptides corresponding to the most intensive peaks in the MALDI-TOF spectrum were determined by tandem (TOF/TOF) mass spectrometry using an ULTRAFLEX mass spectrometer (Bruker Daltonics) in the Department of Proteomic Investigation of the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences. Xyloglucanases of fungal origin, whose amino acid sequences exhibited homology with the indicated peptides, were searched in the SWISS-PROT database using the BLAST2 program (http://cn.expasy.org/tools/ and http://afmb.cnrs-mrs.fr/CAZY/) [12].

The gene of the xyloglucanase of *P. canescens* was isolated and sequenced as described in [11].

Determination of biochemical characteristics of the xyloglucanases. Analytical isoelectrofocusing of proteins

was performed using a Model 111 Cell (Bio-Rad) according to the manufacturer's instructions. SDS-PAGE was performed in 12% polyacrylamide gel using a Mini Protean cell (Bio-Rad). Protein bands were stained with Coomassie Brilliant Blue R-250 (Ferak, Germany). Protein mixtures MW-SDS-200 (30-200 kDa) and IEF-M1A (p*I* 3.6-9.3) (Sigma) were used as the protein standards for SDS-PAGE and isoelectrofocusing, respectively.

Determination of enzymatic activities. Enzymatic activities towards polysaccharide substrates were determined from the initial rates of formation of reducing sugars by the modified Somogyi–Nelson method [13, 14] and by the bicinchoninate method [15]. All indicated activities are expressed as international units per mg protein (U/mg) (one unit corresponds to the amount of the enzyme hydrolyzing 1 μmol glycoside bonds of the substrate per minute).

Kinetic parameters of hydrolysis of specific substrates. Kinetic parameters (K_m, V_{max}) for hydrolysis of tamarind seed xyloglucan by xyloglucanases (50°C, pH 5.0) were determined from the dependence of the initial rates of the hydrolysis on substrate concentration (0.05-20 g/liter) in Lineweaver–Burk coordinates.

Full hydrolysis of xyloglucan. A homogeneous enzyme preparation was incubated with a solution of tamarind seed xyloglucan (5 g/liter) at pH 5.0 (0.1 M sodium-acetate) at 50°C for 3 days. The original concentration of xyloglucanase in the reaction mixture was chosen so as to achieve a 1% decrease in the xyloglucan content after each 5-7 min of the initial reaction phase (this corresponding to the xyloglucanase activity of approximately 0.06 U/ml). During the hydrolysis, aliquots were taken from the reaction mixture, boiled in water-bath for 10 min to stop the reaction, and centrifuged to remove the denatured protein. The resulting samples were assayed for reducing sugars, molecular-mass distribution (MMD) (by high pressure gel permeation chromatography), and qualitative composition of the hydrolysis products (by HPLC using an inserted amino phase column).

Chromatographic assay of products of xyloglucan hydrolysis. Products of the enzymatic hydrolysis of xyloglucan were analyzed using a Chromatography Workstation 700 liquid chromatograph (Bio-Rad). The sugars were detected using a differential refractometer (Knauer, Germany).

Change in the MMD of xyloglucan during the hydrolysis was monitored by high-pressure gel-permeation chromatography on a TSK G3000SW XL column (0.78 × 30 cm; Toso-Haas, Japan). The sugars were eluted with 0.1 M sodium-acetate buffer containing 0.1 M NaCl at flow rate of 0.5 ml/min. The column was calibrated using dextrans (20-250 kDa; Pharmacia).

Composition of low molecular mass products of the hydrolysis of oligo- and polysaccharides was assayed by HPLC analysis [16] on an inserted amino phase Diasorb-130-NH_2 column (6 μm, 0.4 × 25 cm; BioChemMack JSC, Russia) using a 57 : 25 (v/v) acetonitrile–water mixture as the eluent at flow rate of 1 ml/min. The column was calibrated using malto-oligosaccharides (Sigma), isoprimeverose, xylobiose, and laminaritriose (Megazyme), cellobiose (Merck), cellooligosaccharides (polymerization degree of 3-5), and xyloglucan heptasaccharide XXXG (here and further in the text, we used designation suggested by Fry et al. [2]) from our laboratory. The chromatograms were analyzed based on the carbohydrate composition of tamarind seed xyloglucan, namely: 45% glucose, 35% xylose, 16% galactose, and 4% arabinose (http://secure.megazyme.com).

Temperature and pH dependences of xyloglucanase activity. Dependence of the xyloglucanase activity on pH was investigated in 0.2 M citrate-phosphate buffer (pH 2.5-8.0). The temperature dependence was assayed in the range of 4-80°C with an interval of 5°C at pH 5.0. Tamarind seed xyloglucan was used as the substrate.

Stability of xyloglucanases. An enzyme solution (500 μl) was incubated at a certain temperature (40, 50, and 60°C) at pH 5.0 (0.1 M sodium-acetate buffer). After different time intervals (15-60 min), aliquots were taken from the solution, and the activity towards tamarind seeds xyloglucan was determined.

Effect of metal ions on xyloglucanase activity. The xyloglucanase activity was investigated at the presence of Zn^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, and Cu^{2+} (1 mM). A solution of tamarind seed xyloglucan (5 g/liter) was incubated in the presence of the tested effectors for 10 min at 40°C and pH 5.0 (0.05 M sodium-acetate buffer), and then the xyloglucanase activity was determined using the viscosimetric method (by initial rate of decrease in relative viscosity of the tamarind seed xyloglucan solution) [17].

RESULTS AND DISCUSSION

Isolation of homogeneous xyloglucanases from enzyme complexes secreted by *P. canescens* and *P. verruculosum*. The preparation of *P. canescens* PCA10 and *P. verruculosum* B221-151 were separated from non-protein admixtures (insoluble compounds, carbohydrates, pigments, etc.) and then purified by Source 15Q anion-exchange chromatography at pH 6.0 (for PCA10) and at pH 6.2 (for B221-151). In the case of the preparation PCA10, the major part of the xyloglucanase activity (76%) was contained in the first fraction corresponding to the beginning of the NaCl gradient (Fig. 1a). In the case of B221-151, the xyloglucanase activity was found in the 6th and 7th fractions of the salt gradient containing 60 and 30% of the total activity applied to the column, respectively (Fig. 2a). According to the data of SDS-PAGE, these fractions contained a significant amount of other proteins, so they were purified by Superose 12 gel permeation chromatography (PCA10) and Source 15

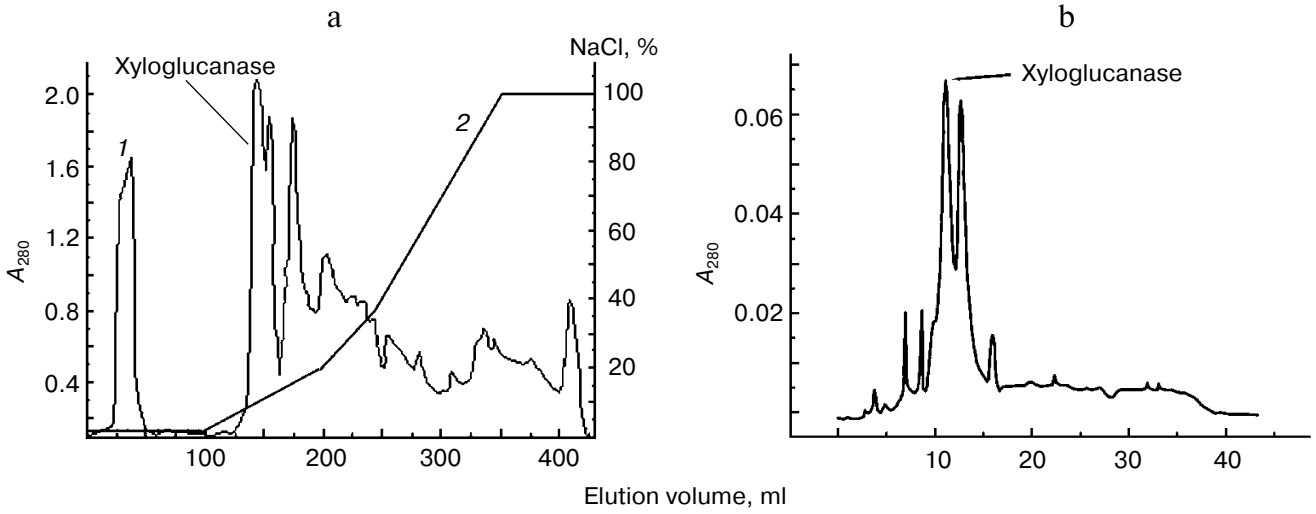

Fig. 1. Isolation of xyloglucanase from the enzyme complex of *P. canescens*: a) anion-exchange Source 15Q chromatography of the crude enzyme preparation at pH 6.0; b) gel-permeation Superose 12 chromatography. *1*) A_{280}; *2*) NaCl gradient.

Isopropyl hydrophobic chromatography (B221-151). During the chromatography of the PCA10 fraction, the major part of the xyloglucanase activity (>70%) was eluted within the 3rd fraction (Fig. 1b) containing a single protein (homogeneity of >95%) of 25 kDa and p*I* 3.5. During the chromatography of the 6th fraction of B221-151 (after Source 15Q), the xyloglucanase activity was found only in fraction A eluted after finishing the ammonium sulfate gradient (Fig. 2b). Chromatography of the 7th fraction of B221-151 (after Source 15Q) resulted in two major fractions of the xyloglucanase activity (B and C) eluted in the middle and at the end of the gradient (Fig. 2c). Fraction B contained a protein of 25 kDa and p*I* 4.1 (>95% homogeneity), and fractions A and C contained a protein of 70 kDa and p*I* 3.5 (>95% homogeneity).

Table 1 presents information on the isolated homogeneous enzymes of *P. canescens* and *P. verruculosum* in comparison with the characteristics of xyloglucanases of *A. japonicus*, *T. reesei*, and *C. lucknowense* [6]. All the xyloglucanases are characterized by the molecular mass of 25-100 kDa, acid p*I* values, and by the absence of isoforms (except for the XG of *T. reesei* that possesses several isoforms of different molecular masses and p*I*). Based on the molecular masses of the xyloglucanases of *P. verruculosum*, the enzymes were named XG 25 and XG 70.

Table 1. Biochemical parameters of fungal xyloglucanases

Producer strain	Enzyme	Molecular mass, kDa	p*I*
P. canescens	XG A	25	3.5
P. verruculosum	XG 25 XG 70	25 70	4.1 3.5
A. japonicus	XG 32	32	2.8
C. lucknowense	XG 78	78	3.8
T. reesei	XG	75-100	4.1-4.3

Search for the proteins homologous to the xylanases of *P. canescens* and *P. verruculosum*. After SDS-PAGE, the protein bands of the xyloglucanases were treated with trypsin, and the resulting hydrolysates were analyzed by MALDI-TOF spectrometry. Analysis of the mass spectra with subsequent search in protein databases MSDB, NCBI, and Swiss-Prot revealed no proteins homologous to the isolated enzymes. To obtain information on the amino acid sequences and subsequent identification, the main peptides (corresponding to the peaks of highest intensity in the MALDI-TOF spectra) of the xyloglucanases of *P. canescens* and *P. verruculosum* were subjected to further fragmentation. Based on the mass spectra of these fragments by TOF-TOF spectrometry, the amino acid sequences of the main peptides were determined. Subsequent analysis of the data with the BLAST2 program using the protein databases revealed xyloglucanases of fungal origin exhibiting homology with the indicated peptides. The xyloglucanase of *P. canescens* and XG 25 of *P. verruculosum* were found to be homologous to the enzymes belonging to the 12th family of glycosyl-hydrolases, while XG 70 of *P. verruculosum* was homologous to the enzymes of the 74th family of glycosyl-hydrolases.

Isolation and sequencing of the gene of *P. canescens* xyloglucanase. The gene encoding the xyloglucanase of *P. canescens* was isolated and sequenced. For this purpose, the nucleotide sequences corresponding to two of four peptides exhibiting the highest intensity in the MALDI-TOF spectra were determined, and PCR primers were

synthesized. The fragment of the genome DNA bearing the gene of xyloglucanase was isolated from the library of *P. canescens* genes cloned into the phage vector [11]. The full sequence of the gene encoding the xyloglucanase was determined (the sequence contained one intron), and based on this sequence, the amino acid sequence of the protein was determined. The xyloglucanase of *P. canescens* contained 251 amino acid residues, and the calculated molecular mass and p*I* value constituted 26.154 kDa and 4.96, respectively. One possible site of N-glycosylation was found in the sequence. The enzyme was identified as xyloglucanase A (gene *xegA*) and designated as XG A.

Substrate specificity of the xyloglucanases was estimated by the specific activities (expressed in activity units per mg of protein) towards the structurally related polysaccharides xyloglucan, β-glucan, and CMC, whose main chain consists of 1,4-β-glucan (or 1,3/1,4-β-glucan in the case of barley β-glucan). Analysis of the influence of the side substituents (in the case of xyloglucan) and carboxymethylation of the glucose residues (in the case of CMC), as well as the presence of the β-1,3-bonds in the backbone (in the case of β-glucan) can help in adequate identification of the enzymes and reveal the difference between them.

The specific activities of the xyloglucanases are presented in Table 2. Among the xyloglucanases of *Penicillium* sp., XG 25 of *P. verruculosum* exhibited the highest activity towards xyloglucan. Among all investigated xyloglucanases, XG A of *P. canescens* exhibited lowest specific activity towards xyloglucan, demonstrating the highest specificity: the enzyme exhibited no activity towards CMC and β-glucan. XG 70 of *P. verruculosum*, XG of *T. reesei*, and XG 78 of *C. lucknowense* were able to hydrolyze β-glucan and CMC (besides the activity towards xyloglucan). However, the efficiency of the hydrolysis of these two substrates was low: the rates of the hydrolysis of β-glucan and CMC were 4- and 40-fold lower (respectively) than that of xyloglucan. The substrate specificity of XG 25 of *P. verruculosum* was close to that of XG 32 of *A. japonicus*.

None of the isolated xyloglucanases of *Penicillium* sp. exhibited activity towards Avicel (microcrystalline cellulose), laminarin, xylan, galactomannan, linear or branched arabinans, polygalacturonic acid, starch, *p*-nitrophenyl derivatives of D-glucose, D-cellobiose, D-galactose, D-xylose, and L-arabinose. In this feature, xyloglucanases of *Penicillium* sp. were similar to XG 32 of *A. japonicus*, XG 78 of *C. lucknowense*, and XG of *T. reesei*.

The hydrolysis of xyloglucan in the presence of xyloglucanases of *Penicillium* sp. was investigated in detail. It was found that although the intensity of full xyloglucan hydrolysis constituted 17-22% for all investigated enzymes, the character of change in MMD and qualitative composition of the products of xyloglucan

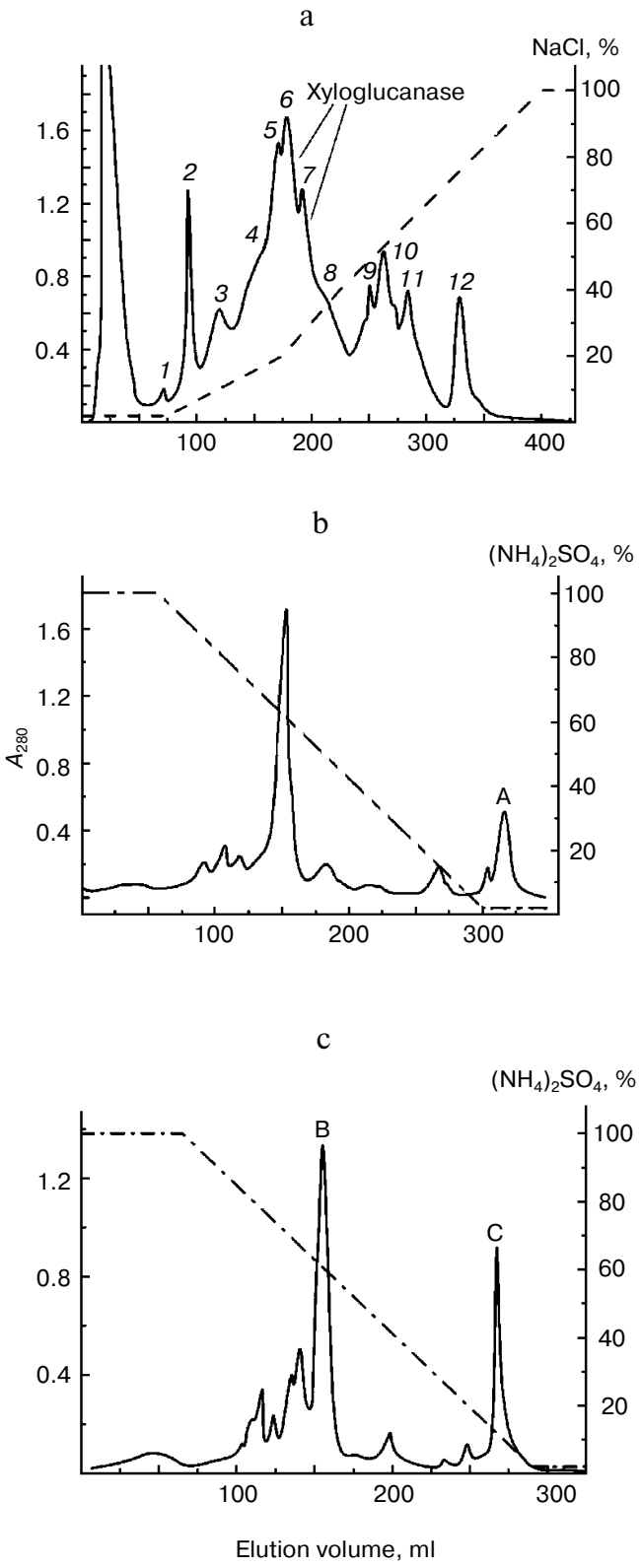

Fig. 2. Isolation of xyloglucanase from the enzyme complex of *P. verruculosum*: a) anion-exchange Source 15Q chromatography of the crude enzyme preparation at pH 6.2; b, c) hydrophobic Source 15 Isopropyl chromatography of fractions 6 (b) and 7 (c). Solid lines, A_{280}; dashed (dot-dashed) lines, salt gradient.

Table 2. Specific activities of xyloglucanases towards polysaccharide substrates (50°C, pH 5.0) determined by the release of reducing sugars using the Somogyi–Nelson method

Producer strain	Enzyme	Activity, U/mg		
		tamarind seed xyloglucan	barley β-glucan	CMC
P. canescens	XG A	21	0	0
P. verruculosum	XG 25 XG 70	52 38	0.2 9.4	0 1.0
A. japonicus	XG 32	98	0.7	0
C. lucknowense	XG 78	75	6.0	3.9
T. reesei	XG	45	3.5	2.6

hydrolysis were different. Analysis of the results on change in MMD of tamarind seed xyloglucan obtained using high-pressure gel-permeation chromatography allowed identification of XG A of *P. canescens* and XG 25 of *P. verruculosum* as endo-depolymerases, since both enzymes quickly decreased the M_r value of the substrate (corresponding to this was the increase in the retention time of the substrate peak with its simultaneous significant widening, Figs. 3a and 3b). (Tamarind seed xyloglucan is a polymer of a high viscosity with a high for polysaccharides M_r value (more than 200 kDa); so, on the chromatogram obtained during the high-pressure gel-permeation chromatography, the peak corresponding to the original xyloglucan was observed within the upper limit of the column range.) XG 32 of *A. japonicus* behaved similarly [6]. In contrast, in the presence of XG 70 of *P. verruculosum*, the M_r value of xyloglucan changed a little with relatively fast growth in the relative content of the low molecular mass products in the reaction mixture (after 48 h of the hydrolysis, the peak area corresponding to the low molecular mass products of the hydrolysis constituted 20% of the total area of the chromatographic profile, Fig. 3c). The similar dependences that were also obtained for XG of *T. reesei* and XG 78 of *C. lucknowense* [6] are characteristic for the enzymes of the mixed catalytic action that is closer to the exo-depolymerase mechanism [18].

Investigation of the composition of the low molecular mass products of full hydrolysis of xyloglucan by HPLC on the inserted amino phase column demonstrated that in the presence of XG A of *P. canescens*, the hydrolysis yielded the only oligosaccharide (peak A on the chromatogram, Fig. 4) with the retention time corresponding to that of xyloglucan heptasaccharide XXXG. In the case of XG 25 of *P. verruculosum* (like xyloglucanases of *A. japonicus*, *T. reesei*, and *C. lucknowense* [6]), three oligosaccharides were formed with different retention time corresponding to peaks A, B, and C eluted after cellopentaose. Tamarind seed xyloglucan is composed of three structural elements of hepta-, octa-, and nonasac-

Table 3. Kinetic parameters for hydrolysis of xyloglucan, β-glucan, and CMC catalyzed by xyloglucanases (pH 5.0, 50°C)

Producer strain	Enzyme	Tamarind seed xyloglucan		Barley β-glucan	
		K_m, g/liter	V_{max}/E_0, sec^{-1}	K_m, g/liter	V_{max}/E_0, sec^{-1}
P. canescens	XG A	1.33 ± 0.12	4.4 ± 0.1	–	–
P. verruculosum	XG 25 XG 70	0.28 ± 0.06 0.29 ± 0.05	50 ± 3 29 ± 2	– 1.32 ± 0.28	– 8.8 ± 2
A. japonicus	XG 32	0.67 ± 0.09	53 ± 2	–	–
C. lucknowense	XG 78	0.31 ± 0.04	78 ± 3	18 ± 3	30 ± 3
T. reesei	XG	0.31 ± 0.06	~55 ± 4	8 ± 2	12 ± 1

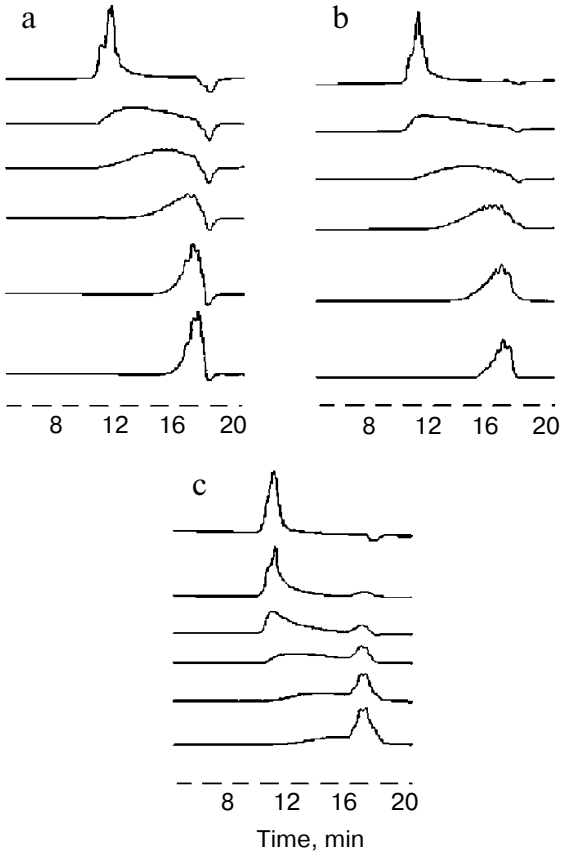

Fig. 3. Gel-permeation chromatography of the products of tamarind seed xyloglucan hydrolysis in the presence of XG A of *P. canescens* (a), XG 25 (b), and XG 70 (c) of *P. verruculosum*. The presented chromatograms correspond to the hydrolysis extent of 0.3, 1.0, 1.4, 1.5, 2.4, and 2.8% in the case of XG A; 0.4, 1.2, 2.3, 4.8, 6.0, and 20% in the case of XG 25; and 0, 1.0, 2.2, 4.3, 5.6, and 8.0% in the case of XG 70.

charides differing from each other by the presence and number of galactose residues β-1,2 bonded to the residues of xylose [1, 3, 4]. Thus, the obtained oligosaccharides A, B, and C with a high probability can be identified as hepta- (XXXG), octa- (XXLG or XLXG), and nonasaccharide (presumably XLLG) differing in the number of galactose substituents.

Hydrolysis of xyloglucan in the presence of XG 70 of *P. verruculosum* yielded octa- and nonasaccharides (B and C), but in significantly smaller amount than in the presence of XG 25. Besides, there was no peak corresponding to heptasaccharide XXXG. The main products of the action of XG 70 on xyloglucan were unidentified oligosaccharides of polymerization degree <5.

Kinetic parameters of the xyloglucanases. K_m values of XG 25 and XG 70 of *P. verruculosum* for tamarind seed xyloglucan hydrolysis were similar (Table 3) and fell within the range that is typical for K_m values for fungal xyloglucanases (0.3-0.7 g/liter). The parameters V_{max}/E_0 determined for xyloglucanases of *P. verruculosum* were also within the range that is typical for fungal enzymes (30-80 sec^{-1}), but the V_{max}/E_0 value for XG 25 almost 1.5-fold exceeded the corresponding parameter for XG 70 (this proportion is also observed for the values of the specific activities of these two enzymes, Table 2). XG A of *P. canescens* differed from other fungal xyloglucanases in a higher K_m value and a lower (approximately 10-fold) V_{max}/E_0 value. This seems to be reasonable considering that among the tested enzymes, XG A exhibited the lowest specific activity towards xyloglucan (Table 2). It should be noted that since XG A exhibited a high K_m value for xyloglucan, the substrate concentration employed for assaying the activity (5 g/liter) was not saturating.

Since XG 70 of *P. verruculosum* was able to hydrolyze β-glucan (Table 2), the parameters K_m and V_{max}/E_0 for this substrate were determined. The V_{max}/E_0 to K_m ratio determined for β-glucan appeared to be 100-150-fold lower than the corresponding parameter determined for xyloglucan. Thus, XG 70 can be also identified as a specific xyloglucanase.

Temperature and pH dependences of the xyloglucanase activity. Dependences of the activity of XG A of *P. canescens* and XG 25 and XG 70 of *P. verruculosum* on pH and temperature are presented in Fig. 5. The enzymes exhibited maximal activity in acidic medium: XG A of *P. canescens* at pH 4.0, and the enzymes of *P. verruculosum*

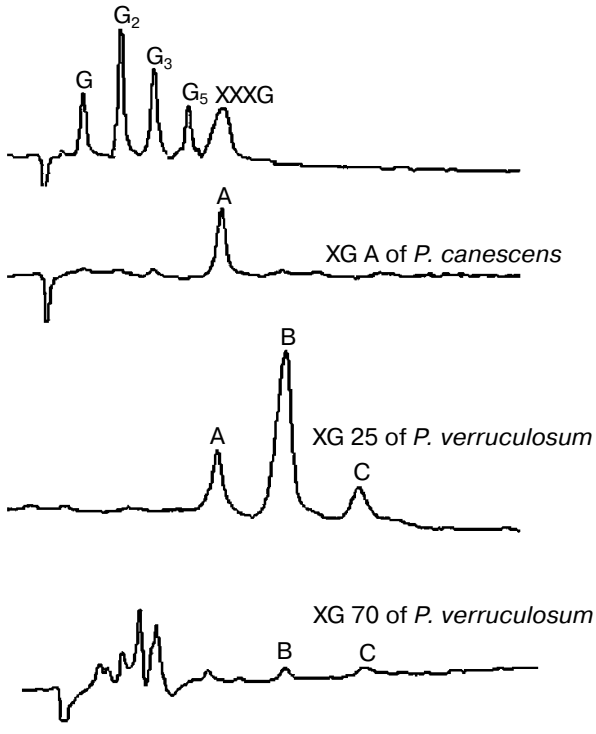

Fig. 4. Composition of the products of tamarind seed xyloglucan hydrolysis in the presence of homogeneous xyloglucanases of *Penicillium* sp. determined by HPLC. G, *D*-glucose; G_2, cellobiose; G_3, cellotriose; G_5, cellopentaose; XXXG, xyloglucan heptasaccharide.

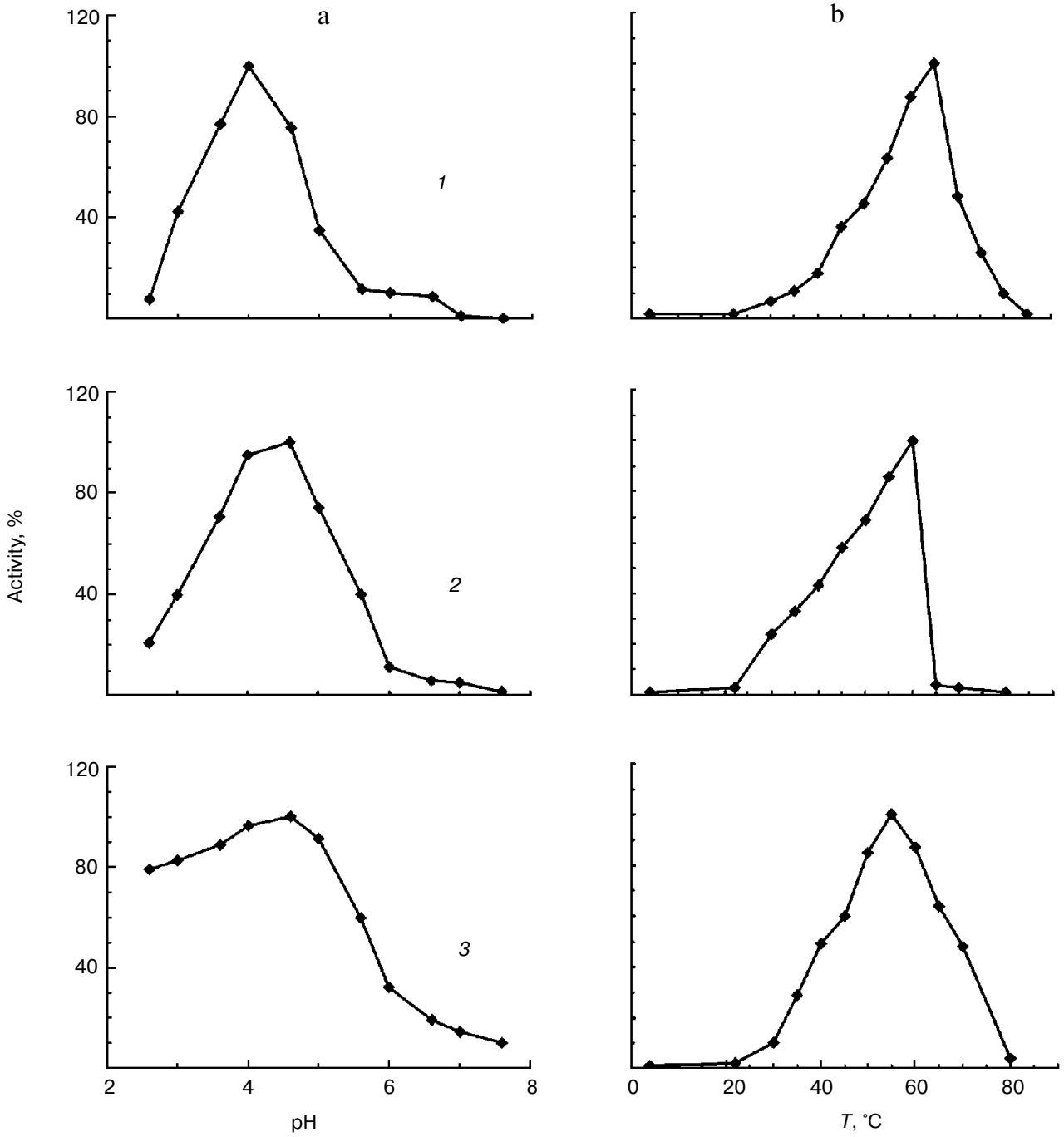

Fig. 5. Dependence of the activity of XG A of *P. canescens* (*1*) and XG 25 (*2*) and XG 70 (*3*) of *P. verruculosum* on pH at 50°C (a) and temperature at pH 5.0 (b) determined by the hydrolysis of tamarind seed xyloglucan.

at pH 4.6. The shapes of the pH profiles of XG A of *P. canescens* and *P. verruculosum* were similar: the activity of both enzymes exceeded 50% in the range of pH 3.2-4.7 (5.5). The pH profile of XG 70 of *P. verruculosum* differed from the two preceding ones: the enzyme exhibited high activity in a strongly acidic pH region, retaining 80% of the original activity at pH 2.5. The xyloglucanases of *A. japonicus* and *T. reesei* exhibited maximal activities in weakly acidic region (pH 5.0), and the pH optimum of XG 78 of *C. lucknowense* was close to neutral values (pH 6.0).

Optimal temperature for the xyloglucanase activity was 65°C for XG A of *P. canescens* and 60°C for XG 25 and 55°C for XG 70 of *P. verruculosum*. In the case of XG 25 of *P. verruculosum*, a sharp drop in the activity was observed already at 65°C (from 100% at 60°C to 5% of the

maximal activity at 65°C), while in the case of XG A of *P. canescens* and XG 70 of *P. verruculosum* the activity gradually decreased with the increase in temperature from 60 to 80°C.

Stability of the xyloglucanases. The ability of the xyloglucanases to retain activity after long-term incubation at elevated temperatures (40-60°C) was investigated at pH 5.0. The xyloglucanases of *Penicillium* sp. were rather stable in solutions at 40 and 50°C, retaining 100% of the original activity after 24 h of incubation. XG 32 of *A. japonicus* behaved similarly, while the other two xyloglucanases taken for comparison were less stable: the half-inactivation time for XG of *T. reesei* and XG 78 of *C. lucknowense* at 50°C was 23 h.

At higher temperatures, the stability of the xyloglucanases significantly decreased. The half-inactivation time for XG 25 of *P. verruculosum* and XG A of *P. canescens* at 60°C was a little more than 1 h, and for XG 70 of *P. verruculosum* it was 45 min. These data indicate that the xyloglucanases of *Penicillium* sp. are situated in the series of stability between XG 32 of *A. japonicus* (half-inactivation time of 28 h at 60°C) and XG of *T. reesei* and XG 78 of *C. lucknowense* that are less resistant to elevated temperatures (half-inactivation time of 20 min at 60°C).

Influence of metal ions on xyloglucanase activity. As known from the literature, fungal enzymes can be sensitive to the presence of bi- and trivalent metal ions in the reaction mixture [3, 4].

The effect of metal ions on the ability of xyloglucanases of *Penicillium* sp. to hydrolyze xyloglucan was investigated in the presence of Cu^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+} salts. In these experiments, the activity was determined by the viscosimetric method, since salts of the mentioned metals can affect the sensitivity of the detection of reducing sugars by the Somogyi–Nelson method. It was found that Cu^{2+}, Fe^{3+}, and Mg^{2+} ions at concentration of 1 mM did not affect significantly the catalytic properties of the xyloglucanases of *Penicillium* sp., while the presence of 1 mM $ZnCl_2$ in the reaction mixture increased the activity by 20%.

REFERENCES

1. Hayashi, T. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **40**, 139-168.
2. Fry, S. C., York, W. S., Albersheim, P., Darvill, A., Hayashi, T., Joseleau, J.-P., Kato, Y., Lorences, E. P., Maclachlan, G. A., McNeil, M., Mort, A. J., Reid, J. S. G., Seitz, H. U., Selvendran, R. R., Voragen, A. G. J., and White, A. R. (1993) *Physiol. Plant.*, **89**, 1-3.
3. Gidley, M. J., Lillford, P. J., Rowlands, D. W., Lang, P., Dentini, M., Crescenzi, V., Edwards, M., Fanutti, C., and Reid, J. S. G. (1991) *Carbohydr. Res.*, **214**, 299-304.
4. York, W. S., van Halbeek, H., Darvill, A. G., and Albershaim, P. (1990) *Carbohydr. Res.*, **200**, 9-13.
5. Pauly, M., Andersen, L. N., Kauppinen, S., Kofod, L. V., York, W. S., Albersheim, P., and Darvill, A. (1999) *Glycobiology*, **9**, 93-100.
6. Grishutin, S. G., Gusakov, A. V., Markov, A. V., Ustinov, B. B., Semenova, M. V., and Sinitsyn, A. P. (2004) *Biochim. Biophys. Acta*, **1674**, 268-281.
7. Peterson, G. L. (1979) *Anal. Biochem.*, **100**, 201-220.
8. James, P. (ed.) (2001) *Proteome Research: Mass Spectrometry*, Springer-Verlag, Berlin.
9. Smith, B. E. (1997) *Protein Sequencing Protocols*, Humana Press, Totowa, 375 p.
10. Yates, J., III (1998) *J. Mass Spectrom.*, **33**, 1-19.
11. Nikolaev, I. V., Bekker, O. B., Serebryanyi, V. A., Chulkin, A. M., and Vinetskii, Yu. P. (1999) *Biotekhnologiya*, **3**, 3-13.
12. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) *Nucleic Acids Res.*, **31**, 3381-3385.
13. Nelson, N. (1944) *J. Biol. Chem.*, **153**, 375-379.
14. Somogyi, M. (1952) *J. Biol. Chem.*, **195**, 19-23.
15. Zorov, I. N., Dubasova, M. Yu., Sinitsyn, A. P., Gusakov, A. V., Mitchenko, A. A., Baraznenok, V. A., Gutyerres, B., and Popova, N. N. (1997) *Biochemistry (Moscow)*, **62**, 704-709.
16. Snyder, L. R., and Kirkland, J. J. (1979) *Introduction to Modern Liquid Chromatography* (2nd Edn.) Wiley-Interscience, N. Y., 863 p.
17. Sinitsyn, A. P., Gusakov, A. V., and Chernoglazov, V. M. (1995) *Bioconversion of Lignocellulose Materials* [in Russian], Moscow State University Publishers, Moscow.
18. Chaplin, M. F., and Kennedy, J. F. (eds.) (1994) *Carbohydrate Analysis: A Practical Approach* (2nd Edn.) Oxford University Press Inc., N. Y., 324 p.