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Isolation and Characterization of Extracellular α-Galactosidases from *Penicillium canescens*

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Abstract—Two α-galactosidases were purified to homogeneity from the enzymatic complex of the mycelial fungus *Penicillium canescens* using chromatography on different sorbents. Substrate specificity, pH- and temperature optima of activity, stability under different pH and temperature conditions, and the influence of effectors on the catalytic properties of both enzymes were investigated. Genes *aglA* and *aglC* encoding α-galactosidases from *P. canescens* were isolated, and amino acid sequences of the proteins were predicted. *In vitro* feed testing (with soybean meal and soybean byproducts enriched with galactooligosaccharides as substrates) demonstrated that both α-galactosidases from *P. canescens* could be successfully used as feed additives. α-Galactosidase A belonging to the 27th glycosyl hydrolase family hydrolyzed galactopolysaccharides (galactomannans) and α-galactosidase C belonging to the 36th glycosyl hydrolase family hydrolyzed galactooligosaccharides (stachyose, raffinose, etc.) of soybean with good efficiency, thus improving the digestibility of fodder.

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Some plant polysaccharides (e.g. pectins and hemicelluloses) contain *D*-galactose residues in both the main and side chains of the polymer molecules. Galactose links also form main chains of galactans and β-1,4-arabinogalactans, as well as side and main chains of β-1,3/1,6-arabinogalactans and galactouronans. Residues of *D*-mannopyranose and *D*-glucopyranose of the main chain of polymer galacto(gluco)mannans (GM)—the major component of hemicelluloses from softwood—are substituted by residues of α-1,6-*D*-galactose. The galactose/glucose/mannose ratio is 1 : 1 : 3 in highly substituted GM and 1 : 1 : 4 in lowly substituted GM [1]. Galactose is a building block of oligosaccharides, such as melibiose, raffinose, stachyose, licheose, and verbascose. The most prevalent galactose-containing plant oligosaccharides are raffinose (Fru(α1,2)-Glu(α1,6)-Gal) and stachyose (Fru(α1,2)-Glu(α1,6)-Gal(α1,6)-Gal) [2].

α-Galactosidases (EC 3.2.1.22) catalyze hydrolysis of terminal non-reducing α-*D*-galactoside bonds. Both natural (galactooligosaccharides, GM, galactolipids) and synthetic (methyl-, ethyl-, phenyl-, *o/p*-nitrophenyl-,

Abbreviations: α-Gal A, α-Gal C) α-galactosidases A and C, respectively; GM) galactomannan; GPC) gel-permeation chromatography; MMD) molecular mass distribution; *p*NP) *p*-nitrophenyl; *p*NPG) *p*-nitrophenyl-α-*D*-galactopyranoside; RS) reducing sugars.

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and 4-methylumbelliferyl-α-*D*-galactopyranosides) α-*D*-galactose-containing compounds can serve as substrates for α-galactosidases [3].

Enzymes effectively hydrolyzing galactooligosaccharides are particularly under consideration when studying α-galactosidases. This is chiefly due to the fact that raffinose and stachyose in soybean and other forage are virtually indigestible for animals, but the treatment of soybean meal with enzyme preparations containing α-galactosidases overcomes this disadvantage of soybean products [4].

α-Galactosidases for which GM are preferential substrates are less studied. Only a few enzymes that effectively hydrolyze side chains of polysaccharides are described in the literature: they are secreted by some representatives of the genus *Penicillium* [5], as well as *Aspergillus niger* [6], *A. tamarii* [7], *Trichoderma reesei* [8], and *Cyamopsis tetragonoloba* [9]. One key property of GM, which determines their usage as food additives, is their ability to form a gel. The treatment of GM with α-galactosidases enables preparation of thickening agents with predetermined rheological properties [10].

The aim of the present work was to isolate *P. canescens* α-galactosidases active against GM and galactooligosaccharides and to study their properties and the possibility and conditions for practical use of α-galactosidases.

MATERIALS AND METHODS

Enzyme preparations. The dry enzyme preparation PCA10 with α-galactosidase activity measured by *p*-nitrophenyl-α-*D*-galactopyranoside (*p*NPG) of 250 U/g prepared on the basis of fungal strain *P. canescens* was obtained from the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino), and the dry α-galactosidase multicomponent preparation α-*D*-Galalactosidase/Amano/10 with the activity of 6000 U/g by *p*NPG was purchased from the Amano Pharmaceutical Co (Japan).

Substrates. The following substrates were used for enzymatic activity determinations. Synthetic substrates included *p*-nitrophenyl glycosides (α-*L*-arabinopyranoside, α-*L*-arabinofuranoside, α-*D*-galactopyranoside, α-*D*-glucopyranoside, β-*D*-glucopyranoside, α-*D*-xylopyranoside, β-*D*-xylopyranoside, α-*D*-mannopyranoside, and β-*D*-mannopyranoside from Sigma (USA)) and oligosaccharides (raffinose and sucrose from Merck (Germany) as well as stachyose from Sigma). Polymeric substrates included larch arabinogalactan (Sigma), potato galactan (Megazyme, Australia), and GM (partially depolymerized soluble acacia GM (gletran) with M_r = 120 and 135 kD and galactose/mannose ratio of 1 : 2.4 and 1 : 1.72, respectively (kindly provided by Dr. V. D. Shcherbukhin)).

Isolation and purification of α-galactosidases. α-Galactosidases from *P. canescens* PCA10 were isolated in three steps: preliminary purification, anion-exchange chromatography, and hydrophobic chromatography. *Penicillium canescens* preparation was preliminarily precipitated with ammonium sulfate (80% saturation at 25°C), re-solubilized in 0.1 M sodium acetate buffer, pH 5.0, and desalted on a column with Acrylex P-2 (Reanal, Hungary) on an Econo-System liquid chromatograph (Bio-Rad, USA) followed by elution with 0.02 M imidazole-HCl buffer, pH 7.65, with flow rate of 1 ml/min. The following purification steps were carried out using an FPLC liquid chromatograph (Pharmacia, Sweden). The column Source 15Q HR 16/5 (Pharmacia) was used for anion-exchange chromatography. The sample containing 100 mg of protein was applied onto the column equilibrated with 0.02 M imidazole-HCl buffer, pH 7.65. The protein bound to the matrix was eluted with buffer containing NaCl of increasing concentration at flow rate of 5 ml/min (gradient volume of 300 ml). The buffer in the accumulated fractions was changed on a column with Bio-gel P4 (Bio-Rad). Hydrophobic chromatography was carried out on Source 15 Isopropyl- (Pharmacia; α-galactosidase A purification) and Phenyl-Superose (Pharmacia; α-galactosidase C purification) columns equilibrated with 1.7 M $(NH_4)_2SO_4$ solution in 0.05 M sodium acetate buffer, pH 5.0. The protein bound to the matrix was eluted with a linearly decreasing gradient of ammonium sulfate at flow rate of 2 ml/min (Source 15 Isopropyl, 240 ml gradient volume) or 0.5 ml/min (Phenyl-Superose, 30 ml gradient volume). The fractions were desalted on a Sephadex G25 (Pharmacia) column with 0.1 M sodium acetate buffer, pH 5.0, as the eluent with flow rate of 0.5 ml/min.

Protein in the samples was determined by Lowry [11] using BSA as a standard or by light absorption at 280 nm.

Isolation and sequencing of α-galactosidase genes. MALDI-TOF mass-spectrometry of α-galactosidase tryptic hydrolyzate was carried out [12-14]. With this aim, a piece of polyacrylamide gel corresponding to α-galactosidase protein band after SDS electrophoresis was treated with trypsin by a method described elsewhere [13]. Gene-modified trypsin for sequencing (Promega, USA) was used at the concentration of 5 ng/ml in 0.05 M NH_4HCO_3 buffer. Peptides were extracted with 20% acetonitrile with the addition of 0.1% trifluoroacetate. MALDI-TOF-mass-spectrometry was carried out at the Department of Proteomic Studies of the Institute for Biomedicine Chemistry of the Russian Academy of Medical Sciences using a REFLEX III spectrometer (Bruker Daltonics, Germany). The NCBI and Swiss-Prot databases were searched using MASCOT software (http://www.matrixscience.com) on the basis of the peptide masses.

P. canescens α-galactosidase genes were isolated and sequenced according to the method described in [15].

BLAST2 software (http://cn.expasy.org/tools/ and http://afmb.cnrs-mrs.fr/CAZY) was used to search for homological proteins [16].

Biochemical characterization of α-galactosidase. The proteins were analyzed by isoelectrofocusing using a Model 111 Cell device (Bio-Rad) according with the manufacturer's protocol. Protein was subjected to electrophoresis under denaturing conditions (in the presence of SDS) in 12% polyacrylamide gel using a Mini Protean cell (Bio-Rad). Protein bands in the gels were stained with Coomassie Brilliant Blue R-250 (Ferak, Germany). The following protein mixtures were used as standards (Sigma): MW-SDS-200 (30-200 kD) for SDS-PAGE and IEF-M1A (p*I* 3.6-9.3) for isoelectrofocusing.

Enzymatic activity determinations. Enzymatic activities with oligo- and polysaccharides as substrates were determined from the initial rates of the formation of reducing sugars (RS) by the modified Somogyi–Nelson method [17, 18] as well as by the bicinchoninate method [19].

Enzymatic activity with synthetic low molecular weight substrates (*p*-NP-glycosides) was determined from the initial rates of the colored reaction product formation at pH 5.0 and 40°C. The enzyme solution (0.1 ml) was injected into a thermostatted plastic tube containing 0.9 ml of the substrate working solution (1 mM in 0.1 M sodium acetate buffer) heated to 40°C. The reaction mixture was agitated 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 coloring of the product formed was determined by spectrophotometry at 400 nm taking into account the substrate, enzyme, and acetate buffer backgrounds determined in reference experiments. The activity was calculated from the molar differential absorption coefficient $\Delta\varepsilon_{400} = 18{,}300\ M^{-1}\cdot cm^{-1}$.

Semiquantitative analysis of numerous samples, in particular those obtained after chromatographic fractionation, was carried out using ELISA 96-well plates. Working solution of the substrate (0.5 mM in 0.1 M sodium acetate, volume of 180 μl) was injected into each well using a multi-channel pipette and incubated at 40°C for 10 min, followed by the addition of the enzyme (20 μl) and rapid agitation of the mixture. The reaction was stopped after 15 min by the addition of 100 μl of 1 M Na_2CO_3. The intensity of the formed coloring was registered using an Anthos Labtec HT2 microplate reader (BioRad) at 405 nm.

All the indicated activities were expressed in international units per mg protein (U/mg, i.e. one unit corresponds to the amount of the enzyme hydrolyzing 1 μmol of glycoside bonds of substrate per minute).

Determination of kinetic parameters of hydrolysis of specific substrates. Kinetic parameters (K_m, V_m) of the action of α-galactosidase on galactose-containing substrates were determined from dependences of the initial hydrolysis rates on the substrate concentrations (0.1-2.5 mM *p*NPG, 0.84-8.41 mM raffinose and stachyose, 0.84-8.41 mM GM) in Lineweaver–Burk coordinates. Constants of α-galactosidase inhibition (K_i) by *D*-galactose were determined from the dependence of the initial *p*NPG hydrolysis rate on the initial substrate concentration (0.05, 0.25, 0.50, 1.0, 2.50, and 27.75 mM) at various concentrations of the inhibitor (0.5, 1.0, 2.5, 5.0, and 10.0 mM for α-galactosidase A and 2.5, 5.0, 15.0, 30.0, and 60.0 mM for α-galactosidase C) at 40°C and pH 5.0.

Exhaustive hydrolysis of GM and oligosaccharides. Homogeneous enzyme was incubated with a solution of GM (5 g/liter) or oligosaccharide (5 g/liter) at pH 5.0 (0.1 M sodium acetate) at 50°C for 24 h. Aliquots of the solution were sampled from the reaction mixture during the hydrolysis process, boiled in a water bath for 10 min to stop the reaction, and centrifuged to remove the denatured protein. The molecular mass distribution of the GM hydrolysis products was determined in the resulting samples by high pressure gel-permeation chromatography (GPC), as well as the concentration of RS was determined, and qualitative composition of low molecular weight galactooligosaccharide hydrolysis products was studied by HPLC on a column with immobilized amino groups.

Study of composition of oligo- and polysaccharide hydrolysis products by chromatography. The composition of products of enzymatic hydrolysis of the oligo- and polysaccharide substrates was analyzed on a Chromatography Workstation 700 (Bio-Rad). The sugars were detected using a differential refractometer (Knauer, Germany).

The changes in molecular mass distribution (MMD) of the polysaccharide substrates during the hydrolysis were monitored by high pressure GPC on a column with TSK G3000SW XL (0.78 × 30 cm; Toso-Haas, Japan). Sodium acetate buffer (50 mM) containing 0.1 M NaCl was used as eluent (flow rate 0.5 ml/min). The column was calibrated with 20-250 kD dextrans (Pharmacia).

The composition of the low molecular weight products of oligo- and polysaccharide hydrolysis was studied by HPLC [20] on a column with immobilized amino groups Diasorb-130-NH_2 (6 μm, 250 × 4 mm; BioKhimMak, Russia). Acetonitrile–water mixture (68 : 32 ratio, flow rate 1 ml/min) was used as eluent, and glucose, galactose, sucrose, raffinose, stachyose, and melibiose solutions at the concentration of 5 g/liter were used as standards for the creation of calibration curves.

Determination of temperature and pH optima for α-galactosidase activity. The following buffer systems were used in the experiments on the determination of pH dependence of α-galactosidase activity: 0.2 M glycine-HCl (pH 2.2-3.6), 0.1 M sodium acetate (pH 3.7-5.6), 0.2 M sodium maleate (pH 5.2-6.8), 0.2 M imidazole-HCl (pH 6.2-7.2), 0.1 M Tris-HCl (pH 7.1-8.9), and 0.2 M glycine-NaOH (pH 8.6-10.6).

Evaluation of α-galactosidase stability. The enzyme solution (0.5 ml) was incubated at a chosen temperature and pH 3-6. Glycine-HCl (0.2 M, pH 3), sodium acetate (0.1 M, pH 4 and 5), and sodium maleate (0.2 M, pH 6) were used to adjust the pH of the medium. The aliquots of the incubated solutions were sampled at chosen time intervals (15-60 min), and enzymatic activity was determined with *p*NPG.

The time intervals between the aliquot sampling were 10-60 sec when studying the α-galactosidase activity under "thermal stress" conditions imitating the enzyme granulation at 80°C for the solutions and wet mixtures, and 10-60 min for dry samples.

Effect of metal ions on the activity of α-galactosidase. Co^{2+}, Ca^{2+}, and Zn^{2+} chlorides, as well as Cu^{2+} acetate at various concentrations were used. The enzyme solution was preliminarily incubated with each of the tested effectors for 15 min at 40°C and pH 5.0 (0.1 M sodium acetate buffer). The α-galactosidase activity was determined according to the standard protocol using *p*NPG as substrate.

Evaluation of ability of α-galactosidase to increase *in vitro* nutritional value of feed for domestic animals and fowl [21]. The method is based on monitoring of composition and concentration of products formed after enzymatic treatment of vegetable feeds. Soybeans were used as a model feed.

Soybean meal was used to study α-galactosidase A. To produce the meal the soybeans were milled using an electric mechanical mill and forced through a sieve with 0.14 mm pores. The meal sample (100 mg) was placed in a 20-ml vessel. Then 2 ml of the enzyme solution in 0.1 M sodium acetate buffer, pH 5.0, was added (dosage of α-galactosidase was calculated by the activity taking 750-18,500 units with *p*NPG per kg of meal). The vessel isolated from moisture was incubated at 37°C under continuous agitation (250 rpm, HAAKE SWB25 thermostatted shaker; Thermo Electron Corporation, USA) for 3 h. Then the reaction mixture was centrifuged (2 min, 12,000 rpm), and the concentration of RS in the supernatant was determined by the Somogyi–Nelson method [17, 18] using the mixture without enzyme as a control.

When studying α-galactosidase C activity, the enzyme preparation (200 μl, 24 activity units with *p*NPG) was added to 1 ml of soybean extract enriched with Soy Solubles galactooligosaccharides (The Solae Company, USA) (the PCA10 preparation was metered by the protein content, i.e. the same amount was taken as in the experiment with α-galactosidase preparations; both preparations were metered by protein content when used simultaneously) and incubated under natural pH conditions (5.5-6.0) and 40°C. The aliquots (200 μl) were sampled from the reaction mixture after 1, 6, 12, 24, and 48 h, diluted 30-50 times with water, and mixed with acetonitrile (acetonitrile/sample 70 : 30). The hydrolysis products were analyzed by HPLC on an analytical column with immobilized amino groups (Diaspher-130-NH_2). The acetonitrile–water mixture (70 : 30) was used as the eluent (flow rate 1 ml/min).

RESULTS AND DISCUSSION

Purification of homogeneous α-galactosidase from the enzyme complex excreted by *P. canescens*. The *P. canescens* preparation PCA10 preliminary purified from non-protein admixtures (non-soluble substances, carbohydrates, pigments, etc.) was subjected to anion-exchange chromatography on a Source 15Q matrix at pH 7.65. α-Galactosidase activity was found in two major fractions eluted by a salt gradient (Fig. 1a). These fractions, by the data of SDS-PAGE, contained a substantial amount of other proteins; therefore, they were subjected to further separation by hydrophobic chromatography. The fraction collected at the beginning of the salt gradient was separated on Source 15 Isopropyl matrix, and the fraction eluted at the end of NaCl gradient on Phenyl-Superose matrix. In both cases, α-galactosidase activity was eluted in a salt gradient as a component of the first major fraction (Fig. 1, b and c). The resulting fractions were additionally purified by gel filtration on Sephadex G25 matrix. Two homogeneous α-galactosidases (by the data of SDS-PAGE and isoelectrofocusing in polyacrylamide gel) with molecular masses of 61 and 80 kD and p*I* 5.1 and 4.8, respectively, were obtained as a result of all purification steps.

Isolation and sequencing of *P. canescens* α-galactosidase genes. Tryptic treatment of α-galactosidase protein bands was carried out after SDS-PAGE, and MALDI-TOF mass-spectra were recorded for the hydrolyzates obtained. Then the amino acid sequences of four peptides with major intensity peaks in MALDI-TOF spectrum were determined for each enzyme by the method of tandem (TOF/TOF) mass spectrometry [12]. The fungal α-galactosidases having homology with these peptides were found in the SWISS-PROT database using the BLAST2 software (http://an.expasy.org/tools/). Then two peptides (in the case of 61-kD α-galactosidase) and three peptides (in the case of 80-kD α-galactosidase) of four were chosen, for which corresponding nucleotide sequences were found and PCR-primers were synthesized. Then two fragments of genome DNA carrying α-galactosidase genes were isolated from *P. canescens* gene library cloned in phage vector [15]. The full-length sequences of genes encoding α-galactosidases were determined (α-galactosidase with molecular mass of 61 kD is encoded by the gene *aglA*, and α-galactosidase with molecular mass of 80 kD is encoded by the gene *aglC*), and amino acid sequences for the proteins containing 417 (*aglA*) and 747 (*aglC*) residues were predicted. Theoretically calculated molecular masses and p*I* of the proteins are: 45,856 and 81,858 daltons, and 5.01 and 4.90, respectively, for 61- and

80-kD α-galactosidases. Five and ten possible glycosylation sites were found in the sequences of 61-kD and 80-kD α-galactosidases, respectively.

Search for proteins homologous to *P. canescens* α-galactosidases. A search with the aid of BLAST2 software demonstrated that the 61-kD α-galactosidase is similar to enzymes related to the 27th glycosyl hydrolase family, and the 80-kD α-galactosidase – to the 36th glycosyl hydrolase family. The *P. canescens* 61-kD and 80-kD α-galactosidases isolated in our work were classified as α-galactosidase A (α-Gal A, *aglA* gene) and α-galactosidase C (α-Gal C, *aglC* gene), respectively.

Substrate specificity of *P. canescens* α-galactosidases. The specific activities of *P. canescens* α-Gal A and α-Gal C to various substrates are given in Table 1. Both enzymes possessed properties typical for the enzymes of their classes. So, the presence of non-reducing α-*D*-galactose group was necessary for expression of α-Gal A and α-Gal C activities. Both enzymes displayed activity exclusively to *p*NPG and were inactive to *p*-nitrophenyl (*p*NP)-derivatives of non-galactose glycosides and β-attached galactopyranoside ring in all synthetic substrates (*p*NP-glycosides), which is evidence for high specificity to the nature and conformation of pyranose ring and glycoside bond type. Note that the specific activity of α-Gal C to *p*NPG is one order of magnitude higher than that of α-Gal A to this substrate.

Both enzymes hydrolyzed natural galactose-containing compounds, besides the synthetic *p*NPG. α-Gal A displayed maximum specific activity to polymeric GM among the studied natural substrates, from which it cleaved α-galactoside side residues. The enzyme hydrolyzed less branched GM (with lower relative galactose content) most effectively, which can apparently be explained by steric obstacles. Note that α-Gal A did not display its activity to polymeric substrates such as arabinogalactan and galactan containing galactose in the main chain of the polymer molecule. The character of MMD changes of polymeric GM was studied during the hydrolysis by α-Gal A. It has been demonstrated that the average molecular mass of these substrates alters insignificantly with the comparably rapid growth of the relative content of low molecular weight products in the mixture under the action of α-Gal A (the area of peak corresponding to the low molecular weight hydrolysis products comprised 20-40% of the total area of the chromatography profile at 24 h (Fig. 2)), which is typical for exodepolymerases [22]. The depth of GM exhausting hydrolysis by α-Gal A was relatively high comprising 45 and 55% for highly and lowly substituted GM, respectively, and *D*-galactose was the only low molecular weight product (by the data of HPLC on the column with immobilized amino groups).

Unlike α-Gal A, α-Gal C did not hydrolyze polymeric galactose-containing substrates, but it was highly specific to galactooligosaccharides (the α-Gal C activity

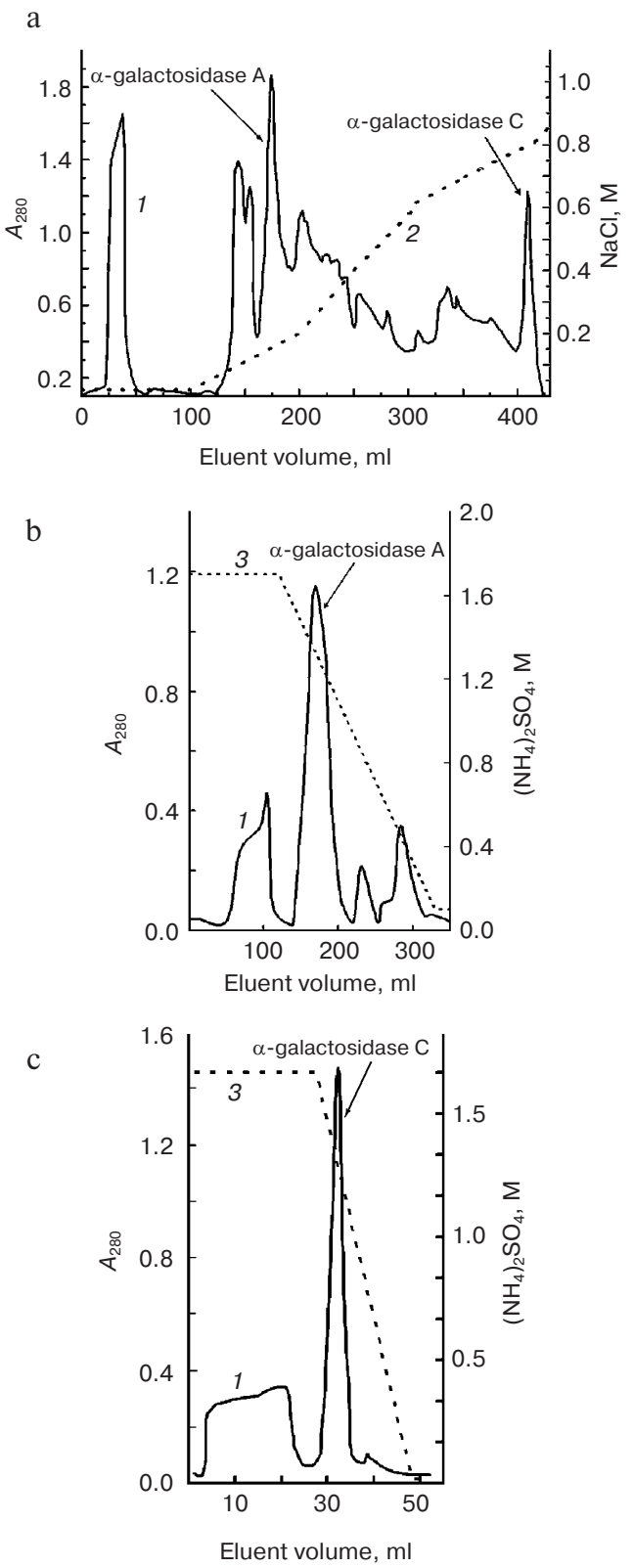

Fig. 1. Anion-exchange chromatography of the initial *P. canescens* PCA10 enzyme complex on Source 15Q matrix at pH 7.65 (a) and hydrophobic chromatography on Source 15 Isopropyl (b) and Phenyl-Superose (c). *1*) Protein, A_{280}; *2*, *3*) concentration gradients of NaCl and $(NH_4)_2SO_4$, respectively.

values with these substrates are more than two orders higher than that for α-Gal A). Neither of the α-galactosidases can hydrolyze sucrose, whose molecule does not contain terminal α-*D*-galactoside group, but preferred those substrates among galactooligosaccharides whose carbohydrate chain contains lesser amount of residues: the specific activities of α-Gal A and α-Gal C decreased by 60 and 10%, respectively, when raffinose was substituted by stachyose. The analysis of composition of the galactooligosaccharide hydrolysis products by HPLC on the column with immobilized amino groups demonstrated that both α-galactosidases hydrolyze raffinose (although with different efficacy) to form the "sucrose + galactose" pair as the product. Gradual hydrolysis to the pair raffinose + galactose was observed in the case of stachyose. Then gradual hydrolysis of raffinose to the sucrose + galactose pair occurred (Fig. 3). Transglycosylation products were not detected under the conditions of this experiment.

Table 1. Specific activities of *P. canescens* α-Gal A and α-Gal C

Substrate	Activity, U/mg	
	α-Gal A	α-Gal C
	pH 5.0, 40°C	
*p*NP-α-*D*-galactopyranoside	98	1255
*p*NP-β-*D*-galactopyranoside	0	0
*p*NP-α-*D*-arabinopyranoside	0	0
*p*NP-α-*D*-arabinofuranoside	0	0
*p*NP-α-*D*-mannopyranoside	0	0
*p*NP-β-*D*-mannopyranoside	0	0
*p*NP-α-*D*-xylopyranoside	0	0
*p*NP-β-*D*-xylopyranoside	0	0
*p*NP-β-*D*-glucopyranoside	0	0
*p*NP-α-*L*-rhamnopyranoside	0	0
*p*NP-α-*L*-fucopyranoside	0	0
Sucrose	0	0
Raffinose	1.2	296
Stachyose	0.52	256
	pH 5.0, 50°C	
GM I*	6.2	0
GM II**	3.5	0
Non-branched arabinan	0	0
Galactan	0	0
Arabinogalactan	0	0
Arabinoxylan	0	0
Arabinan	0	0

* Galactomannan with galactose/mannose ratio of 1 : 2.4 (120 kD).
** Galactomannan with galactose/mannose ratio of 1 : 1.72 (135 kD).

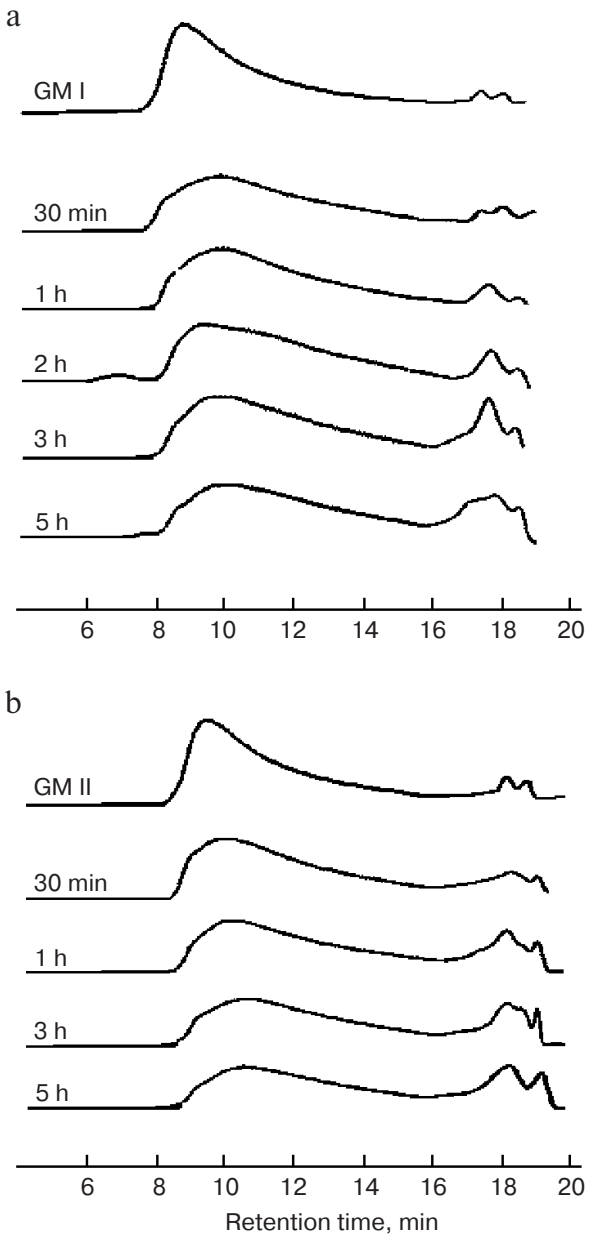

Fig. 2. Chromatograms of the GM hydrolysis products under the action of *P. canescens* α-Gal A obtained by gel-permeation chromatography. a, b) GM with galactose/mannose ratio of 1 : 2.4 (120 kD) and 1 : 1.72 (135 kD), respectively.

Kinetics parameters of *P. canescens* α-galactosidases. These parameters are shown in Table 2. High k_{cat} values are typical for α-Gal C in the hydrolysis of low molecular weight galactose-containing substrates, more than three orders exceeding the corresponding values for α-Gal A, and located on the highest boundary of k_{cat} values typical for α-galactosidases from other sources from the 36th family glycosyl hydrolases [4, 6, 23, 24]. On the other hand, the K_m values of oligosaccharide hydrolysis by α-Gal C were two orders lower than the correspon-

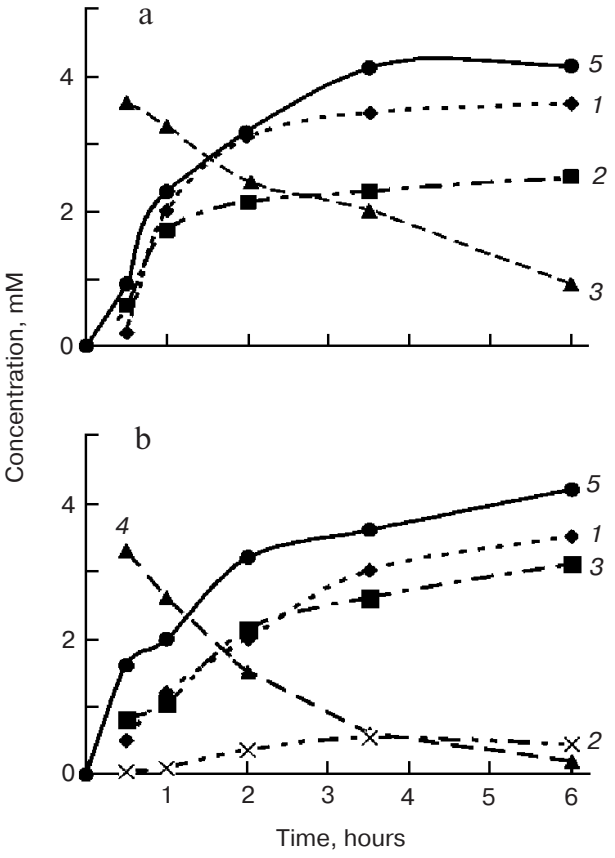

Fig. 3. Kinetics curves (obtained by HPLC) quantitatively describing the alteration in the reaction mixture composition during the hydrolysis of raffinose (a) and stachyose (b) by α-Gal C from *P. canescens*: *1*) galactose; *2*) sucrose; *3*) raffinose; *4*) stachyose; *5*) RS.

ding values found for α-Gal A. As mentioned above, when comparing the specific activities of the two enzymes, α-Gal A, unlike α-Gal C, "prefers" high molecular weight galactose-containing compounds as substrates (k_{cat} of GM hydrolysis is one order higher than k_{cat} of oligosaccharide hydrolysis). A comparison of kinetics parameters for α-Gal A determined with polymeric GM with the literature data seems to be not correct, because these values depend on physical and chemical characteristics of polysaccharide substrates, which, in turn, vary depending on the natural source of polysaccharides, method of their isolation, and other conditions.

Temperature and pH dependences of α-Gal A and α-Gal C activities. Dependences of α-Gal A and α-Gal C activities on pH and temperature are given in Fig. 4. Profiles of pH dependence for the two α-galactosidases are very similar. Both α-Gal A and α-Gal C displayed maximum activity in acidic medium (at pH 4.0-5.0 and 5.0, respectively). The difference was that α-Gal C maintained notable activity in the alkaline region (pH 6-8; the activity of α-Gal A was decreased to zero at pH 7) and α-Gal A – in the acidic region (pH 2.5-3.0; the activity of α-Gal C was decreased to zero at pH 2.5).

Temperature profiles of activity for the two α-galactosidases are similar as well, but temperature optima for the enzymes are different (optimum temperatures for α-Gal A and α-Gal C are 55 and 65°C, respectively). Note that acidic pH optimum and temperature optimum between 45-60°C are typical for the majority of known fungal α-galactosidases [4, 6, 21, 25, 26].

Stability of α-Gal A and α-Gal C. The ability of α-galactosidases to maintain their activity after prolonged elevated temperature (40-60°C) was studied at pH 3-6.

Maximum stability of α-Gal A activity was observed at pH 4-5 in the temperature range 40-50°C (the enzyme maintained no less than 90% of its initial activity after 3 h incubation under these conditions). The stability of α-Gal A was slightly decreased at pH 3 and 6 (the enzyme retained 80 and 70% of its initial activity after 3 h incubation at 40 and 50°C, respectively). The elevation of temperature to 60°C negatively influenced the stability of α-

Table 2. Kinetics parameters of *p*NPG, GM, and galactooligosaccharide hydrolysis catalyzed by *P. canescens* α-Gal A and α-Gal C

Substrate	K_m		k_{cat}, sec^{-1}	
	α-Gal A	α-Gal C	α-Gal A	α-Gal C
*p*NPG (40°C, pH 5.0)	0.48 ± 0.05 mM	0.61 ± 0.05 mM	7 ± 1	1800 ± 250
Raffinose (40°C, pH 5.0)	0.49 ± 0.06 mM	23 ± 2 mM	0.89 ± 0.05	1100 ± 100
Stachyose (40°C, pH 5.0)	0.22 ± 0.04 mM	21 ± 2 mM	0.57 ± 0.02	1250 ± 80
GM I (50°C, pH 5.0)*	34 ± 3 μM	–	10 ± 1	–
GM II (50°C, pH 5.0)**	44 ± 3 μM	–	7 ± 1	–

* Galactomannan with the galactose/mannose ratio of 1 : 2.4 (120 kD).
** Galactomannan with the galactose/mannose ratio of 1 : 1.72 (135 kD).

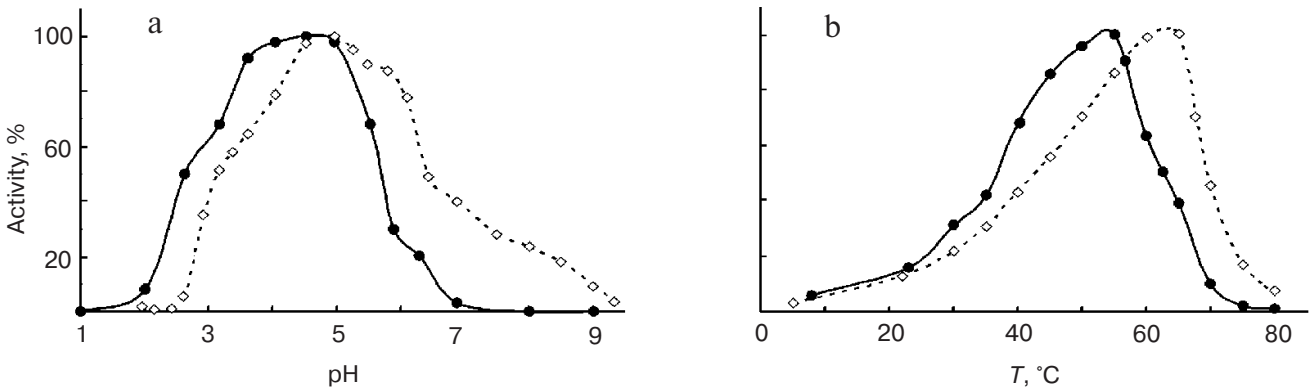

Fig. 4. Dependences of α-Gal A activities (closed circles) and α-Gal C (open rhombi) of *P. canescens* on pH (at 40°C) and temperature (at pH 5.0) ((a) and (b), respectively) determined by hydrolysis of *p*NPG.

Gal A (half-life periods of the enzyme were 40 min for pH 5, 30 min for pH 4, and 15 min for pH 3 and 6).

α-Gal C was more stable than α-Gal A in moderately acidic and neutral media and maintained no less than 95% of its initial activity after 3 h incubation at 40-50°C and pH 4-6. Much higher stability of α-Gal C should be noted at 60°C and pH 5 (the enzyme retained 90% of its initial activity after 3 h incubation under these conditions). However, the stability of α-Gal C was also decreased with the decrease of pH to 3. The enzyme retained 80% of its initial activity after 3 h incubation at 40°C, but it became completely inactivated for the same time range when the temperature increased to 50°C (half-life period – 1 h). The half-life period of α-Gal C was 10 min at 60°C and pH 3 (although this value was 2 h at pH 4).

The study of α-Gal A and α-Gal C behavior at granulation temperatures (80°C) demonstrated that dry forms of the enzymes are very resistant to short-time effect of high temperatures: both enzymes retained 100% of their initial activities for 5 min under humidity no more than 5% (they retained 100% of their initial activity for several hours under zero-humidity conditions). The stability of the enzymes decreased with increase of humidity: so, α-Gal A retained 80%, and α-Gal C – 20% of their initial activities after 5 min incubation under 10% humidity; the half-life period for both enzymes was 80 sec under 20% humidity.

Inhibition of α-Gal A and α-Gal C by the reaction product, D-galactose. It is known that the hydrolysis reaction product (*D*-galactose) is generally an inhibitor of α-galactosidase activity, wherein the inhibition type can

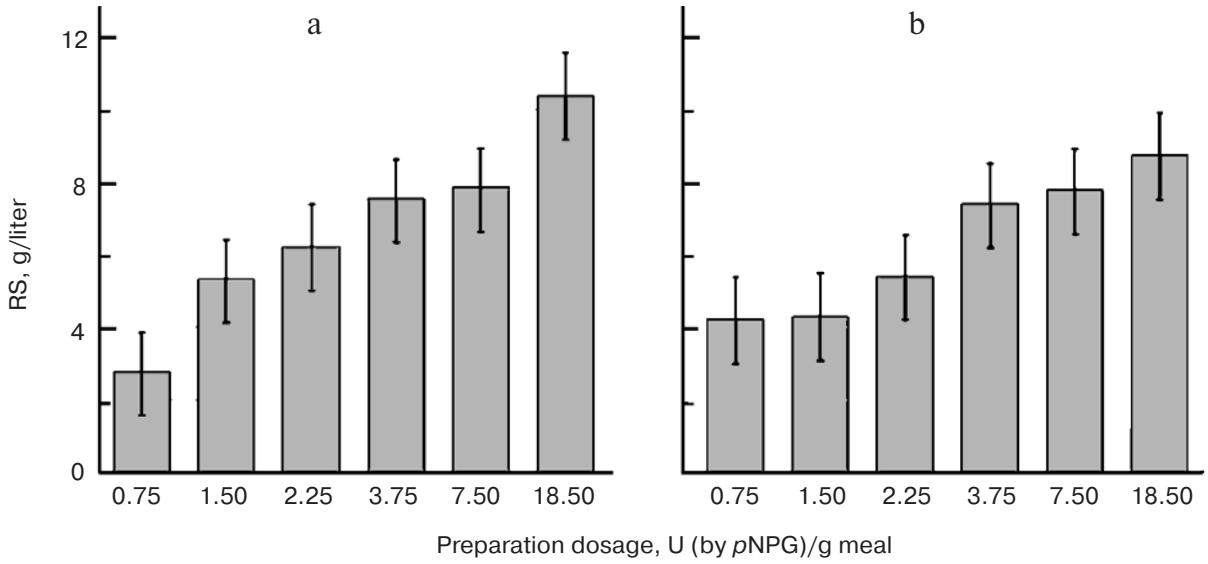

Fig. 5. Dependence of RS yield on the dosage of the enzyme preparation upon hydrolysis of soybean meal: *1*) homogeneous α-Gal A from *P. canescens*; *2*) commercially available α-galactosidase preparation α-*D*-Galalactosidase/Amano/10.

vary [4, 6, 7, 25-28]. We have found that 50% of the initial activity of *P. canescens* α-Gal A is lost in the presence of 1.25 mM *D*-galactose. α-Gal C was one order less sensitive to the presence of the reaction product in the reaction mixture (28% of its activity was lost in the presence of 28 mM *D*-galactose). The study of dependence of kinetics parameters of *p*NPG hydrolysis reaction on concentration of *D*-galactose in the reaction medium demonstrated that *D*-galactose is a competitive inhibitor for both α-galactosidases ($K_i = 1.0 \pm 0.3$ and 30 ± 5 mM for α-Gal A and α-Gal C, respectively).

Effect of metal ions on the activities of α-Gal A and α-Gal C. The majority of known α-galactosidases are sensitive to the presence of mono- and divalent metal ions in the reaction medium [7, 23, 28, 29]. We studied the effects of Co^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+} on the ability of α-Gal A and α-Gal C to hydrolyze *p*NPG, a specific substrate. We found that Co^{2+}, Ca^{2+}, and Zn^{2+} (at their concentrations up to 0.1 M) have no effect on the catalytic properties of α-Gal A, whereas Cu^{2+}, when present in the reaction medium at the concentration of 0.1 M, causes the decrease of the activity by 20%. α-Gal C is also not inhibited by 0.1 M solutions of Co^{2+} and Ca^{2+}, but Zn^{2+} taken at this concentration causes the decrease in enzymatic activity by 80%, and 0.02 M Cu^{2+} completely inactivates α-Gal C.

Similar results (inhibition by cupric ions) were described, for instance, for α-galactosidases from soybeans [23] and some α-galactosidases of fungal origin [7, 28].

Evaluation of abilities of α-Gal A and α-Gal C to fortify *in vitro* feed for domestic animals and fowl. The main sources for vegetable proteins in feeds for domestic animals and fowl are soybean, maize, pea, and other leguminous crops. GM and galactooligosaccharides are components of leguminous crops (for instance, soybean consists of ~0.5% GM and ~6% galactooligosaccharides). These substances are "anti-nutrient" factors: GM counteracts the activity of digestive enzymes, and galactooligosaccharides cannot be utilized by the organism, but fermented by intestinal microflora causing the formation of gases and diarrhea. The use of enzyme preparations containing α-galactosidase can solve these problems [30-32].

A number of *in vitro* tests of the enzymes were carried out to evaluate the usefulness of *P. canescens* α-galactosidases as additives to feeds when soybean and products of its processing were used as substrates. The commercially available α-*D*-Galalactosidase/Amano/10 biological additive preparation was used as a "positive control". The efficacy of homogeneous α-Gal A was evaluated by the yield of RS in the solution after 3 h of incubation (the time range which is required for food passing through the digestive tract of the animal) of the enzyme with soybean meal at 37°C (the animals' body temperature). As seen from Fig. 5, *P. canescens* α-Gal A was similar to the α-*D*-Galalactosidase/Amano/10 preparation in the ability to decompose soybean meal with the formation of RS.

The efficacy of homogeneous α-Gal C was evaluated by the extent of conversion of galactooligosaccharide-enriched soybean extract "Soy Solubles" containing 35.3% mono- and oligosaccharides including 2.8% raffinose, 12% stachyose, 19% sucrose, 1.5% fructose, and 0.3% glucose and galactose, by the data of HPLC. The composition of sugars in the control experiment in the absence of the enzyme was unchanged in time (chromatograms obtained at the initial time point and after 48 h were identical). Peaks of sucrose, stachyose, and raffinose were present on the chromatography profile (Fig.

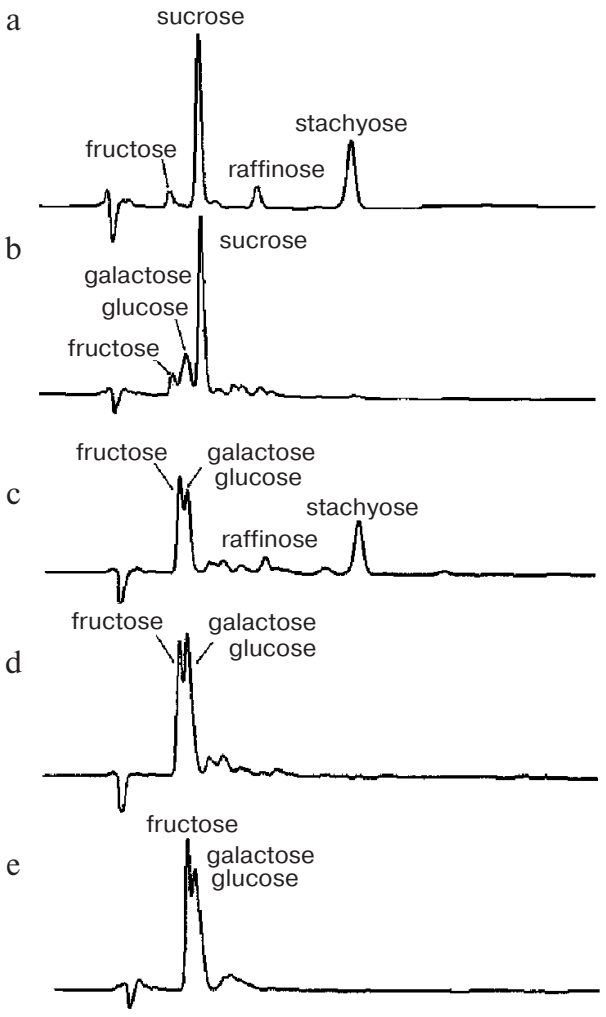

Fig. 6. Composition of the hydrolysis products of concentrated galactooligosaccharide-enriched soybean extract "Soy Solubles" under the action of homogeneous α-Gal C from *P. canescens* (b), *P. canescens* PCA 10 preparation (c), mixture of homogeneous α-Gal C and *P. canescens* PCA 10 preparation (d), commercial preparation of α-*D*-Galalactosidase/Amano/10 (e) determined by HPLC; a) the enzymatically untreated substrate.

6a). The peaks of stachyose and raffinose disappeared, and the content of galactose was substantially increased under the action of α-Gal C after 48 h of the hydrolysis (Fig. 6b). The α-*D*-Galalactosidase/Amano/10 commercial preparation hydrolyzed all galactooligosaccharides including sucrose after 48 h of incubation (Fig. 6e). This is due to the presence of invertase activity in the preparation: sucrase hydrolyzes sucrose to glucose and fructose. The enzyme preparation PCA10 prepared from the strain *P. canescens* possessed a remarkable invertase activity and very low total α-galactosidase activity. Therefore, the peaks of stachyose and raffinose are virtually the same as for control on the chromatogram reflecting the effect of *P. canescens* PCA10, but sucrose was completely hydrolyzed to fructose and galactose (Fig. 6c). The exhaustive hydrolysis of all galactooligosaccharides to fructose and galactose occurred under the common action of α-Gal C and *P. canescens* PCA10 preparation on soybean concentrate, wherein the resulting chromatogram (Fig. 6d) is similar to the chromatogram of the commercial preparation α-*D*-Galalactosidase/Amano/10.

Thus, two novel α-galactosidases (α-Gal A and α-Gal C excreted by the mycelial fungal strain *P. canescens*) have been isolated and characterized, and the genes encoding α-Gal A and α-Gal C (*aglA* and *aglC*, respectively) have been isolated and sequenced. The ability of α-galactosidase for effective hydrolysis of extracted fraction of feed thus providing its fortification has been demonstrated (with soybean as an example).

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