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Isolation and Properties of Recombinant Inulinases from *Aspergillus* sp.

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Abstract—The genes *inuA* and *inu1*, encoding two inulinases (32nd glycosyl hydrolase family) from filamentous fungi *Aspergillus niger* and *A. awamori*, were cloned into *Penicillium canescens* recombinant strain. Using chromatographic techniques, endoinulinase InuA (56 kDa, p*I* 3) and exoinulinase Inu1 (60 kDa, p*I* 4.3) were purified to homogeneity from the enzymatic complexes of *P. canescens* new transformants. The properties, such as substrate specificity, pH- and T-optima of activity, stability at different temperatures, influence of cations and anions on the catalytic activity, etc., of both recombinant inulinases were studied.

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Inulin (polyfructan) serves as a reserve polysaccharide in more than 30,000 plant species, the majority of which are bilobular and belong to the Compositae and Campanulacea families (for example, topinambour, artichoke, chicory, dahlia) [1]. The inulin molecule is a linear polymer chain consisting of β-2,1-linked fructose (F) monomers with a glucose (G) monomer as the end part [2]. The polymerization degree (PD) of fructose in an inulin molecule GF_n can vary from 3 to 60 with a mean value of 32-34.

Inulin is hydrolyzed by two enzymes – exoinulinase (β-*D*-fructan fructohydrolase, EC 3.2.1.80), and endoinulinase (2,1-β-*D*-fructan fructanohydrolase, EC 3.2.1.7). Endoinulinases hydrolyze internal bonds of the inulin polymer forming inulotriose (nistose (GF_3)), inulotetraose (fructosil-1-nistose (GF_4)), inulopentaose, etc. as the main reaction products [3]. Exoinulinases hydrolyze terminal β-2,1-fructoside bonds of inulin and sucrose [4, 5]. All fungal inulinases known to date belong to the 32nd family of glycosyl hydrolases (http://afmb.cnrs-mrs.fr/CAZY/index.html) [6].

Inulinases are used in production of high-fructose syrups [7], inulooligosaccharides (fructooligosaccharides, FOS, with PD 2-9 [8]) synthesis, and a wide spectrum of microbiological synthesis products: bioethanol, butanediol, lactic and citric acids, etc. [9]. Moreover, fructose contributes to assimilation of iron by forming chelate complexes with it [10]. Inulooligosaccharides are also used in obtaining functional and therapeutic products as prebiotics [11]. The demand for inulinases in food industry requires preparing effective inulinase enzyme samples (both exo- and endodepolymerases).

Lower fungi from the *Aspergillus* genus are natural producers of inulinases. It is known, for example, that the InuA inulinase is secreted by a strain of *A. niger*, and the Inu1 exoinulinase is produced by a strain of *A. awamori* [12, 13], but the productivity of these strains is not very high.

In the Laboratory of Enzyme Biotechnology (Bach Institute of Biochemistry, Russian Academy of Sciences),

Abbreviations: CL, culture liquid; CMC, carboxymethylcellulose sodium salt; FOS, fructooligosaccharides; GPC, gel permeation chromatography; HPLC, high performance liquid chromatography; Inu, inulinase; MMD, molar mass distribution; PD, polymerization degree; RS, reducing sugars.

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Penicillium canescens fungus is used as a laboratory model for obtaining producer strains of heterologous and homologous target enzymes. Compared to other fungi, this strain has a number of advantages: it has a higher culture growth rate and faster biosynthesis of extracellular enzymes and possesses a well-studied system of expression and transformation of exogenous DNA [14].

The goal of this work was to study new enzyme samples from *P. canescens* strains with heterologous expression of exo- and endoinulinases from *Aspergillus* fungi, and to isolate and analyze the properties of the recombinant proteins.

MATERIALS AND METHODS

Enzyme preparations. We used dry enzyme preparations obtained by cool dehumidification of *P. canescens* A3 recombinant strain culture liquid (CL) with a heterologous InuA endoinulinase gene from *A. niger*, *P. canescens* A12 recombinant strain CL with a heterologous Inu1 exoinulinase gene from *A. awamori* strain, as well as CL of control recipient *P. canescens* RN3-11-7 strain obtained in a 10-liter fermenter in medium consisting of sugar beet pulp (3%), peptone (3%), and KH_2PO_4 (2.5%). Fermentation was conducted for 120 h at 28°C and pH 4.5-5.0. An equivalent medium was used for fermentation in shaker flasks.

Substrates. The following substrates were used for evaluation of enzyme activities: inulin from topinambour (Reakhim, Russia), chicory (Sigma, USA), agave (Dyadic, USA), and artichoke (Sigma) and oligosaccharides raffinose, sucrose (Merck, Germany), and stachyose (Sigma).

Isolation and purification of inulinases. Inulinases were isolated from *P. canescens* samples in three stages: preliminary purification of samples, anion-exchange chromatography of the complex, and hydrophobic chromatography. Samples were preliminarily precipitated by ammonium sulfate (80% saturation at 25°C) and resuspended in 0.1 M Na-acetate buffer, pH 5.0. They were desalted on a Biogel-P4 column (BioRad, USA) using an Econo-System (BioRad) liquid chromatograph with 0.02 M Bis-Tris/HCl eluent, pH 6.8, with flow rate of 1 ml/min. Following stages of purification were conducted using an FPLC (Pharmacia, Sweden) liquid chromatograph. A Source 15Q HR 16/5 (Pharmacia) column was used for anion-exchange chromatography. A sample containing 100 mg of protein was loaded on the column equilibrated with 0.02 M Bis-Tris/HCl, pH 6.8. Protein bound on the column was eluted in a NaCl gradient from 0 to 0.4 M with flow rate of 5 ml/min (volume of gradient 300 ml). The buffer in collected fractions was exchanged on column with Biogel-P4 (BioRad).

Hydrophobic chromatography of fractions which expressed inulinase activity was conducted on column with Source 15 Isopropyl (Pharmacia) sorbent equilibrated with 1.7 M $(NH_4)_2SO_4$ in 0.05 M Na-acetate buffer, pH 5.0. Protein bound on the column was eluted by buffer with linearly decreasing concentration of ammonium sulfate with flow rate of 2 ml/min (volume of gradient 240 ml). The fractions were desalted conducted on a column with Sephadex G-25 sorbent (Pharmacia); the eluent was 0.1 M Na-acetate buffer, pH 5.0; 0.5 ml/min flow rate.

The content of protein in samples was determined by Lowry et al. protein assay [15] using BSA as standard or by optical absorption of samples at 280 nm.

Identification of inulinases using mass spectrometry. Inulinases were identified by a standardized technique [16]. Mass-spectra of hydrolysates were recorded using a REFLEX III instrument (Bruker Daltonics, Germany) in the Department of Proteomic Studies of the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences. The determined peptide masses were compared with theoretically calculated molecular masses of putative peptides, which were obtained from known amino acid sequences of *A. niger* InuA and *A. awamori* Inu1.

Determination of biochemical properties of recombinant inulinases. Analytical isoelectric focusing of proteins was conducted on Model 111 Cell (BioRad) according to the device manual. Electrophoresis of proteins under denaturing conditions (in the presence of SDS) was conducted in 12% polyacrylamide gel on Mini Protean (BioRad) apparatus. Proteins were stained by Coomassie brilliant blue R-250 (Ferak, Germany). MW-SDS-200 (30-200 kDa) and IEF-M1A (p*I* 2.80-6.55) (Sigma) protein mixtures were used as standards for SDS-PAGE and isoelectric focusing, respectively.

Activity of recombinant inulinases. The activities of recombinant inulinases toward inulins from different plants (topinambour, chicory, artichoke, etc.) and oligosaccharide substrates (sucrose, raffinose, stachyose) were measured by the initial rates of reducing sugar (RS) formation by the modified Somogyi–Nelson method [17, 18]. The activity was expressed in international units per mg protein (one unit of activity corresponds to the amount of enzyme which hydrolyzes 1 μmol of substrate glycoside bonds in 1 min).

Complete hydrolysis of inulin by recombinant inulinases. Homogeneous enzyme was incubated with inulin solution (50 g/liter) in 0.1 M Na-acetate buffer, pH 5.0, at 50°C for 2 days. The initial concentration of inulinases in the reaction mixture was selected to obtain 1% inulin hydrolysis per each 5-7 min of the initial stage of reaction (which corresponds to the average inulinase activity of 0.06 unit/ml). During the progress of the hydrolysis, aliquots of solution were taken from the reaction mixture, heated in water bath for 10 min to stop the reaction, and centrifuged to remove denatured protein. RS concentration was estimated in the samples, as well as molar mass distribution (MMD) determined by high-pressure gel-

permeation chromatography (GPC) and the qualitative composition of inulin hydrolysis products by HPLC on a column with bound amino phase.

Chromatographic analysis of composition of inulin hydrolysis products. The composition of inulin enzymatic hydrolysis products was analyzed with a liquid Chromatography Workstation 700 (BioRad). Sugar was determined with a differential refractometer (Knauer, Germany).

Changes in the MMD of inulin during hydrolysis were tracked with high-pressure GPC on a Bio-Gel TSK 30 XL (0.78 × 30 cm; BioRad, Japan) column. A 0.1 M Na-acetate buffer, pH 5.0, containing 0.1 M NaCl and 0.05% NaN_3 was used as the eluent (flow rate 0.46 ml/min). The column was calibrated with dextrans (20-250 kDa; Pharmacia).

The composition of low molecular weight inulin hydrolysis products was estimated by HPLC on a Diaspher-110-Amine column with bound amino phase (5 μm, 0.4 × 25 cm; VSM, Russia). A mixture of acetonitrile and water with 75 : 25 volume ratio was used as the eluent (flow rate 1 ml/min). Glucose (Chelicon, Russia), fructose, and sucrose (Merck) were used as standards.

RESULTS AND DISCUSSION

Microorganism strains. The recipient strain *P. canescens* RN3-11-7 (niaD$^-$) was used for plasmid transformation. The strain is auxotrophic in the *niaD*$^-$ gene, which is responsible for synthesis of nitrate reductase. An *A. awamori* strain was used for isolating genome DNA, which was used as the template for the amplification of the *inu1* gene encoding exoinulinase (Inu1). An *Aspergillus niger* strain was used for isolating genome DNA, which was the template for amplification of the *inuA* gene encoding endoinulinase (InuA). The results of the *P. canescens* RN3-11-7 strain plasmid transformation were *P. canescens* InuA_Anig and *P. canescens* Inu1_Awa plasmid transformants.

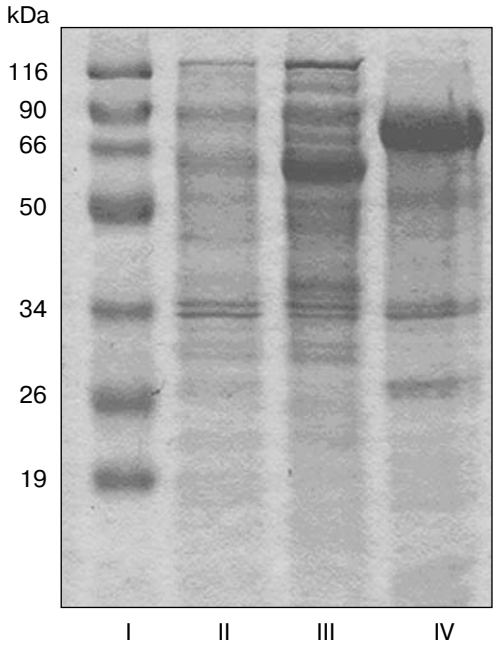

Fig. 1. SDS-PAGE of CL secreted by new transformants. I, standard markers for SDS-PAGE; II, enzyme complex secreted by the recipient strain *P. canescens* RN3-11-7; III, enzyme complex secreted by *P. canescens* InuA_Anig strain; IV, enzyme complex secreted by of *P. canescens* Inu1_Awa strain.

Electrophoretic analysis of enzyme samples and results of mass spectrometry. The analysis of the CL obtained with new transformants by SDS-PAGE (Fig. 1) revealed that compared to the enzyme complex of the *P. canescens* RN3-11-7 recipient strain, in the case of the *P. canescens* Inu1_Awa strain a significant increase in the protein band was observed in the region of 60 kDa (apparently corresponding to Inu1 exoinulinase), and in the case of the *P. canescens* InuA_Anig strain a significant increase was observed in the 56 kDa protein band (apparently corresponding to InuA endoinulinase). These protein bands were excised, treated with trypsin, and the hydrolysates were analyzed by MALDI-TOF mass spectrometry. Analysis of the mass spectra and subsequent comparison with the theoretically calculated molecular masses of the putative peptides (determined from known amino acid sequences of *A. niger* InuA and *A. awamori* Inu1) confirmed that the 56 and 60 kDa bands correspond to *Aspergillus* sp. endo- and exoinulinases, respectively.

Clones having maximum target activity were used for fermentation in shaker flasks, leading to selection of two transformants: A3 (from the *P. canescens* InuA_Anig series of transformants) and A12 (from the *P. canescens* Inu1_Awa series of transformants), which provided 250 and 2900 units/ml inulinase activity in CL, respectively (compared to 2 units/ml for the *P. canescens* RN3-11-7 recipient strain). The activity in CL was measured on topinambour inulin. Both of the selected strains, A3 and A12, were used for fermentation in a 10-liter fermenter. Dry enzyme samples of *P. canescens* A3 and *P. canescens* A12 were obtained, which contained the recombinant InuA endoinulinase from *A. niger* and the Inu1 exoinulinase from *A. awamori* with inulinase activity (measured on topinambour inulin) of 2750 and 21,300 units/g with protein level of 235 and 245 mg/g of sample, respectively.

Isolation of recombinant endo- and exoinulinase from enzyme complexes secreted by *P. canescens*. *Penicillium canescens* A3 and A12 samples were preliminarily purified from non-protein impurities (insoluble substances, carbohydrates, pigments, etc.) and subjected to anion-exchange chromatography on Source 15Q, pH 6.8. In the case of the *P. canescens* A3 sample, inulinase activity was expressed by a major fraction in the middle of the basic NaCl gradient (0.15-0.20 M NaCl; Fig. 2a), and in case

of the *P. canescens* A12 sample the activity on inulin was expressed by a major fraction in the end of the basic NaCl gradient (0.25-0.35 M NaCl; Fig. 3a). SDS-PAGE showed that these fractions contained a significant amount of other proteins, so they were subjected to further separation by hydrophobic chromatography on Source 15 Isopropyl. During the separation of the endoinulinase fraction, the main part of the desired enzyme passed out in the third fraction of the ammonium sulfate gradient (Fig. 2b), which contained one protein (degree of homogeneity >95%) with a molecular weight of 56 kDa and p*I* 3. During the separation of the exoinulinase fraction the required activity was observed in the first fraction at the beginning of the ammonium sulfate gradient (Fig. 3b). These fractions contained a protein

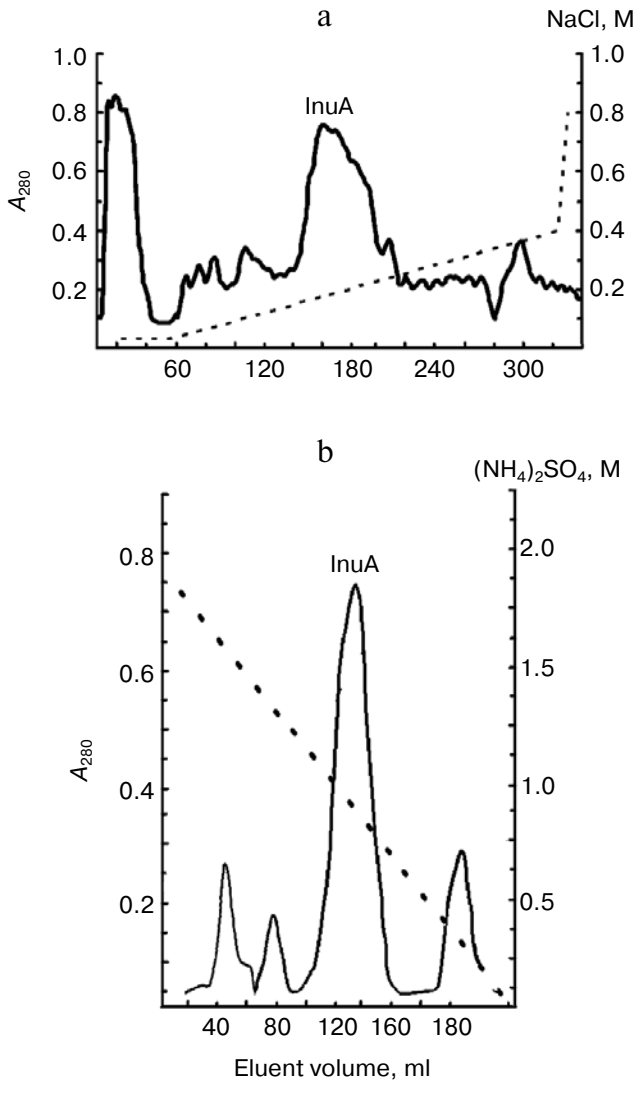

Fig. 2. Isolation of endoinulinase InuA. a) Anion-exchange chromatography on Source 15Q of *P. canescens* A3 enzyme complex at pH 6.8; b) hydrophobic chromatography on Source 15 Isopropyl of inulinase fraction. Solid curves, A_{280}; dashed lines, gradient of salt concentration.

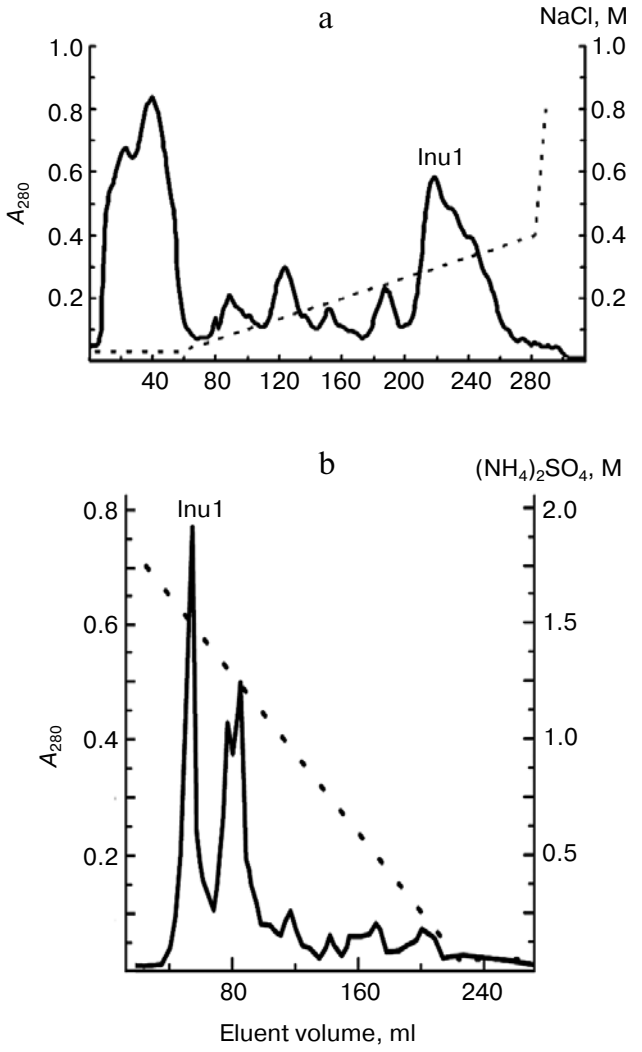

Fig. 3. Isolation of exoinulinase Inu1. a) Anion-exchange chromatography on Source 15Q of *P. canescens* A12 enzyme complex at pH 6.8; b) hydrophobic chromatography on Source 15 Isopropyl of inulinase fraction. Solid curves, A_{280}; dashed lines, gradient of salt concentration.

with a molecular weight of 60 kDa, p*I* 4.3 (degree of homogeneity >95%).

Substrate specificity of the inulinases. Activities of the homogeneous inulinases with respect to different substrates are shown in Table 1. Endoinulinase showed the greatest activity among polysaccharide substrates on inulins from topinambour and artichoke, the activity on agave inulin (the most branched substrate) being 5-10 times lower. Exoinulinase, by contrast, was most active on agave inulin and showed, respectively, 3 and 4 times lower activity on the inulins from artichoke and topinambour (their molecules have a more linear structure).

Exoinulinase Inu1, as expected, displayed activity on oligomeric substrates containing fructose substitute on the non-reducing end of the carbohydrate molecule. With increasing degree of polymerization of the oligomeric

Table 1. Specific activities of inulinases (pH 5.0, 50°C)

Substrate	Activity, units/mg protein*	
	exoinulinase (Inu1)	endoinulinase (InuA)
Inulin of:		
agave	325	2.2
chicory	90	32
artichoke	10	10
dahlia	70	28
topinambour	80	31
Sucrose (Glc-Fru)	80	8
Raffinose (Gal-Glc-Fru)	495	0.5
Stachyose (Gal_2-Glc-Fru)	180	0.4

* Specific activity of enzyme preparations was measured by RS yield according to the Somogyi–Nelson method [17, 18].

molecule (in the series sucrose–raffinose–stachyose), the enzyme activity decreased. Endoinulinase showed no detectable activity on the these oligosaccharides.

Mechanism of inulinase activity in relation to inulins from different sources. Analysis of data on changes in MMD of inulins from different sources caused by Inu1 and InuA inulinases, conducted with GPC, confirmed, respectively, exo- and endodepolymerase mechanisms of their activity. Specifically, during prolonged hydrolysis of inulin by exoinulinase Inu1 a similar pattern was observed for all substrates – a slight decrease in the area of the high molecular weight peak corresponding to the initial substrate, without any changes in retention time of this peak, with simultaneous increase in the area of the peak corresponding to the low molecular weight reaction products (Fig. 4). The degree of exhaustive hydrolysis under the influence of exoinulinase was 65, 75, 80, and 90% for inulins from chicory, artichoke, agave, and topinambour, respectively. The only low molecular weight product of hydrolysis in all cases was fructose.

Hydrolysis efficiency of endoinulinase InuA was maximal in the case of artichoke inulin, which has almost linear structure (conversion degree was close to 100%), as well as for topinambour and chicory inulin (degree of their exhaustive hydrolysis was 75 and 80%, respectively). Endoinulinase demonstrated the ability to quickly reduce the average molecular weight of these substrates (it is shown on the chromatogram as an increase in the retention time of the substrate peak with simultaneous broadening) with the formation of oligomeric products (Fig. 5). Analysis of low molecular weight fractions formed during the hydrolysis of inulins from artichoke, chicory, and topinambour under the influence of endoinulinase by HPLC on a column with bound amino phase showed that their composition depends on the degree of conversion of the substrate (Fig. 6). For example, when the degree of conversion was ⩽30%, the main products of hydrolysis were tri- and tetramers; with degree of conversion of ~50% they were mono-, di-, and trimers; with degree of conversion of 70% or more hydrolysis produced monomers (fructose). In the case of agave inulin having a more branched structure, the maximum degree of hydrolysis was 15-20%, the average molecular weight of the substrate (retention time) changed only slightly, and the area of the peak corresponding to low molecular weight reaction products grew relatively slowly (Fig. 5b). The only low molecular weight hydrolysis product was fructose.

Thus, the exhaustive hydrolysis of inulin from different sources by either exo- or endoinulinase led to the formation of fructose as a final product, demonstrating high, although somewhat variable depending on the inulin source, conversion degree of the substrate. Inulin was hydrolyzed by exoinulinase by a consecutive processing mechanism, sequentially splitting off terminal fructose monomer units, and hydrolyzing the terminal bond between fructose and glucose. Endoinulinase hydrolyzed internal β-2,1-fructoside bonds of inulin in the early stages of inulin hydrolysis, leading to initial formation of oligosaccharides and their subsequent conversion to fructose. It should be noted that the presence of branch points in agave inulin made it not possible for endoinulinase to provide a high degree of exhaustive hydrolysis of the substrate.

Hydrolysis of agave inulin with mixtures of exo- and endoinulinases. Since the efficiency of hydrolysis of agave inulin with individual endoinulinases was low, a mixture of exo- and endoinulinases was used for the hydrolysis of this substrate.

With simultaneous addition of exo- and endoinulinases into the reaction mixture containing agave inulin, the total yield of RS compared to individual action of endoinulinase increased, mainly due to increase in the yield of fructose. Analysis of low molecular weight end products by HPLC on a column with bound amino phase showed that they consisted of only fructose and disaccharide. The final concentration of oligomeric products did not exceed 3 g/liter (which corresponds to a hydrolysis degree of ~6%), and their yield was virtually independent of exoinulinase concentration in the reaction mixture. Therefore, in simultaneous use of endo- and exoinulinases, the latter is aggressive toward the substrate even at low concentrations and forms the final fructose product, bypassing the intermediate oligomeric products.

Inulinases were added successively to the reaction mixture containing agave inulin: in the first stage, exoinulinase Inu1 was introduced into the mixture, then, after a certain period of time (1, 2, 4, or 6 h after hydrolysis) endoinulinase InuA was added to the partially hydrolyzed substrate (degree of conversion of which, determined from RS yield, was 6, 10, 13, or 17%, respectively). Half

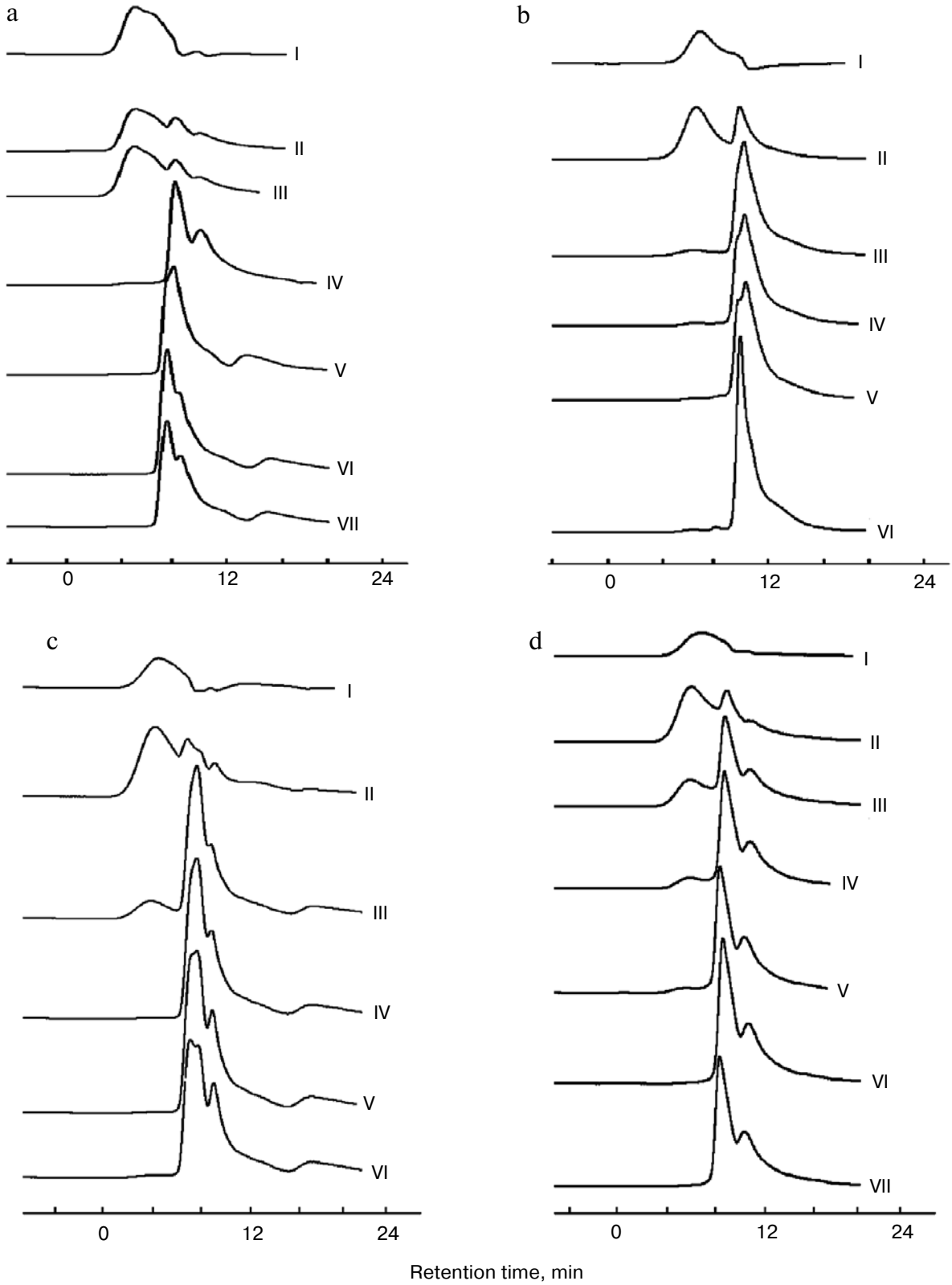

Fig. 4. Separation of hydrolysis products of inulins from artichoke (a), chicory (b), topinambour (c), and agave (d) after Inu1 treatment by GPC. Chromatograms correspond to hydrolysis degrees of 15 (II), 20 (III), 60 (IV), 73 (V), 74 (VI), and 75% (VII) in the case of artichoke inulin; 20 (II), 40 (III), 50 (IV), 60 (V), and 65% (VI) in the case of chicory inulin; 20 (II), 40 (III), 50 (IV), 70 (V), and 90% (VI) in the case of topinambour inulin, and 10 (II), 25 (III), 35 (IV), 70 (V), 75 (VI), and 80% (VII) in the case of agave inulin. I, chromatogram of the substrate.

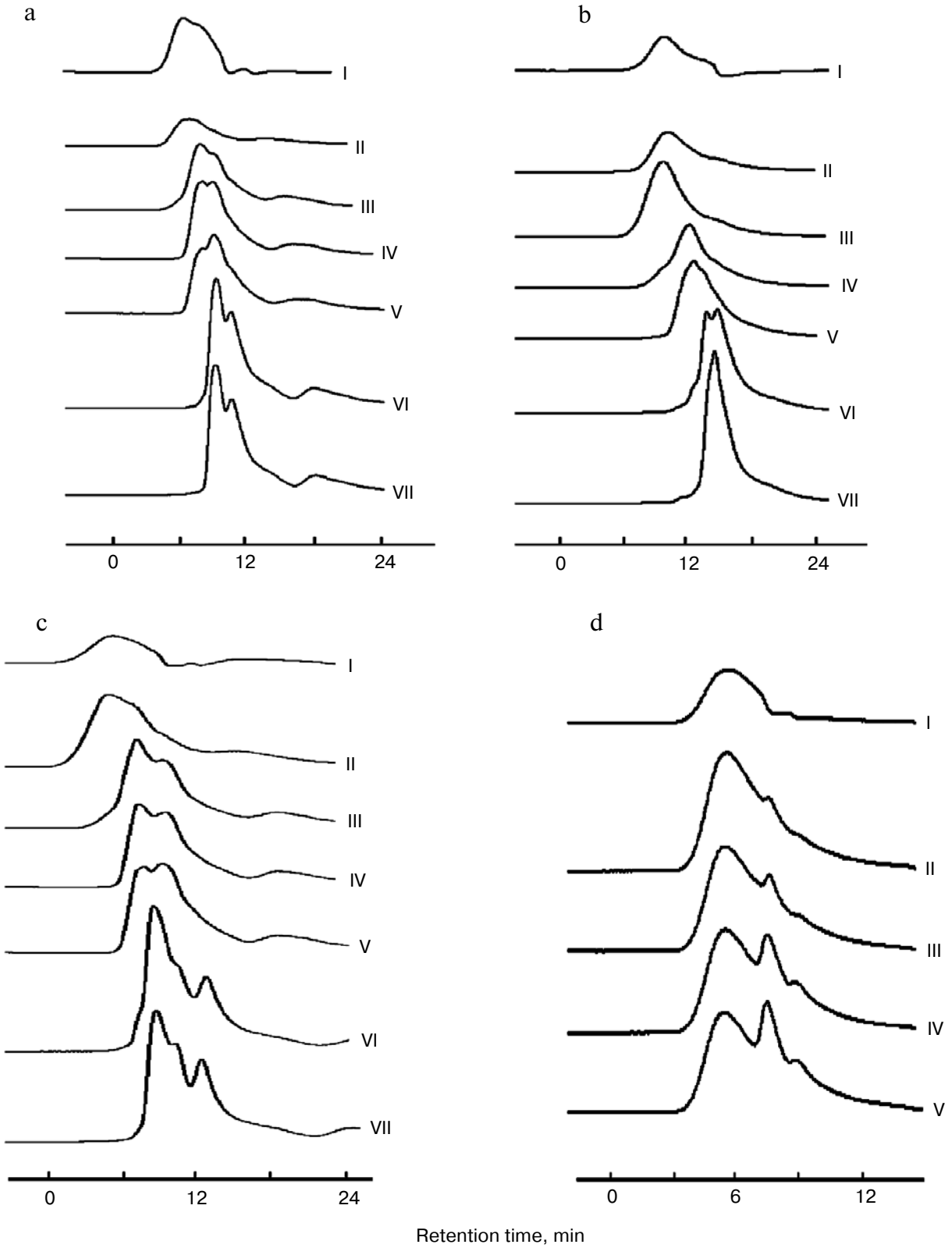

Fig. 5. Separation by GPC of hydrolysis products for inulins of artichoke (a), chicory (b), topinambour (c), and agave (d) after InuA treatment. Chromatograms correspond to hydrolysis degrees of 3 (II), 20 (III), 30 (IV), 45 (V), 95 (VI), and 100% (VII) in the case of artichoke inulin; 4 (II), 7 (III), 10 (IV), 15 (V), 50 (VI), and 80% (VII) in the case of chicory inulin; 4 (II), 9 (III), 15 (IV), 40 (V), 55 (VI), and 75% (VII) in the case of topinambour inulin, and 2 (II), 3 (III), 8 (IV), and 15% (V) in the case of agave inulin. I, chromatogram of the substrate.

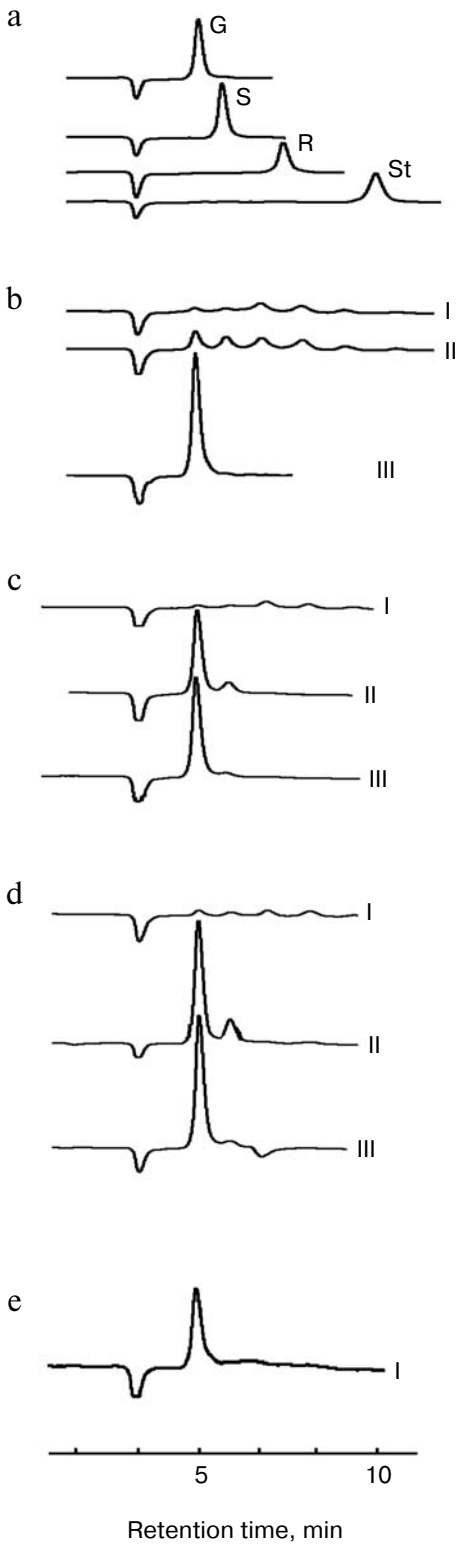

Fig. 6. Composition of inulin hydrolysis products after InuA treatment determined by HPLC. a) Standard sample (G, *D*-glucose; S, sucrose; R, raffinose; St, stachyose). b) Artichoke at hydrolysis degrees of 20 (I), 45 (II), and 100% (III); c) chicory at hydrolysis degrees of 10 (I), 50 (II), and 80% (III); d) topinambour at hydrolysis degrees of 15 (I), 55 (II), and 75% (III); e) agave at hydrolysis degrees of 15% (I).

of all samples, formed after the first stage of hydrolysis, were subjected to high temperature treatment to inactivate exoinulinase. It was found that the final concentration of oligomeric products, representing a mixture of isomers of inulobiose and inulotriose, after sequential introduction of exo- and endoinulinases to the reaction mixture was more than two times higher than the yield of oligosaccharides after simultaneous introduction of the enzymes. The final yield of oligosaccharides was practically independent of the inulin hydrolysis degree in the first stage of hydrolysis and amounted 6.5-6.8 g/liter provided Inu1 was inactivated after the first stage of hydrolysis and 6.2-6.5 g/liter without inactivation of the exoinulinase. However, inactivation of exoinulinase influenced the content of fructose in the final products: without inactivation the fructose content was 2-3 times higher than with it. Therefore, production of fructooligosaccharides (as prebiotics) from agave inulin by InuA treatment only require a brief pre-processing of the substrate by Inu1, and it is necessary to carry out the inactivation of exoinulinase to reduce the content of fructose in the hydrolysate.

Kinetic parameters of inulinases. K_m values of topinambour inulin hydrolysis by exo- and endoinulinases were 26.5 and 12.5 g/liter, respectively (Table 2), which is in the range of K_m values characteristic of fungal inulinase (1-30 g/liter) [19]. The values of k_{cat} for recombinant InuA and Inu1 also were in the typical range for exo- and endoinulinases of fungal origin (0.6-2.7 sec^{-1}), and the value of k_{cat} for exoinulinase was almost 1.5-3.0 times higher than for endoinulinase. There was a high value of k_{cat} of sucrose hydrolysis by Inu1.

pH and temperature dependence of inulinase activity. Dependences of recombinant InuA and Inu1 activity on pH and temperature are shown in Fig. 7. Exoinulinase Inu1 demonstrated maximal activity in acidic medium at pH 4.0-4.5, while endoinulinase InuA was most active at pH ~ 6. The forms of pH profiles of Inu1 and InuA were different: Inu1 showed $\geqslant$50% activity at pH $\leqslant$ 6.5, and InuA demonstrated the same activity at 4.7 $\leqslant$ pH $\leqslant$ 7.2. It should be also noted that Inu1 was very active in the strongly acidic pH region, retaining about 80% of the maximum activity at pH 3.0. The temperature optimum of the enzyme activity was 50°C for Inu1 and 55°C for InuA. Inu1 showed $\geqslant$50% activity at 27°C $\leqslant$ T $\leqslant$ 60°C, and InuA – at 30°C $\leqslant$ T $\leqslant$ 70°C.

Stability of inulinases. The ability of exo- and endoinulinase to remain active during long-term exposure to elevated temperatures (40-70°C) was studied at pH 5.0 (for InuA and Inu1) and 7.0 (for InuA). In acidic solutions InuA was more stable than Inu1: endoinulinase retained 100, 95, and 75% of the initial activity at 40, 50, and 60°C, respectively, after 3 h of incubation at pH 5.0, whereas for exoinulinase these parameters were 85, 70, and 3%, respectively (half-inactivation time of Inu1 at 60°C and pH 5.0 was 7 min). With temperature increase

Table 2. Kinetic parameters of hydrolysis reactions of inulins from different sources catalyzed by InuA and Inu1 (pH 5.0, 50°C)

Substrate	Exoinulinase Inu1		Endoinulinase InuA	
	K_m, g/liter	k_{cat}, sec^{-1}	K_m, g/liter	k_{cat}, sec^{-1}
Inulin of:				
topinambour	27 ± 2	2.7 ± 0.2	12 ± 1	1.7 ± 0.1
dahlia	25 ± 2	2.3 ± 0.2	15 ± 1	0.83 ± 0.07
chicory	11 ± 1	1.2 ± 0.1	4.9 ± 0.3	0.60 ± 0.05
Sucrose	8.1 ± 0.5	11 ± 1	–	–

to 70°C, the stability of both enzymes in solution decreases: the half-inactivation time of InuA was 5 min and that of Inu1 was 3 min at pH 5.0. Thermal inactivation in solutions at pH 6.5-7.0 was also investigated for InuA, whose pH optimum lies in the neutral zone. It was found that the enzyme is less stable at neutral and slightly alkaline pH than in weakly acidic medium: half-inactivation times of endoinulinase at pH 6.5-7.0 were 240, 180, 4, and 2 min at 40, 50, 60, and 70°C, respectively.

The search for possible effectors that stabilize Inu1 and InuA solutions revealed that additions of sucrose, sorbitol, and NaCl have a positive effect on the stability of these enzymes. Thus, inulinases retained 80 and 75% activity in a solution containing sucrose (20%), NaCl (5%), and sorbitol (0.1%) after 14 days of incubation at 25 and 40°C, respectively (in the absence of stabilizers InuA lost 30 and 75% and Inu1 lost 45 and 90% of the initial activity at 25 and 40°C, respectively).

Effect of metal cations and acid anions on the activity of inulinases. It is known that enzymes of fungal origin may be sensitive to the presence of bi- and trivalent metal ions (which are present in raw vegetable stock) in the reaction mixture, as well as to some anions of organic and inorganic acids [20, 21].

The effect of metal ions on the ability of the recombinant exo- and endoinulinases to hydrolyze inulin was tested with salts of Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+}, and Zn^{2+}. We used the viscometric method in this experiment to measure the activity, since salts of these metals may affect the sensitivity of activity determination based on the Somogyi–Nelson RS detection method. It was found that Na^{+} has no appreciable effect on the catalytic properties of the studied inulinases. On the other hand, Fe^{3+}, Mn^{2+}, and Ni^{2+} demonstrated strong inhibitory effect both on Inu1 and InuA (0.05 M solutions of salts of these metals almost completely inhibited both enzymes). Similar effect was shown by 0.15 M solutions of Co^{2+}, Mg^{2+}, and Zn^{2+}. It is interesting to note that Ca^{2+} exerted a negative effect only on exoinulinase: 0.05, 0.15, and 0.25 M solutions of Ca^{2+} salts decreased the activity of Inu1 by 15, 40, and 80%, respectively. In contrast, with respect to endoinulinase Ca^{2+} behaved as an activating agent: the maximum activation effect (increase in activity by 40%) was observed in the presence of 0.025 M Ca^{2+}, and it disappeared in the presence of 0.15 M Ca^{2+} (0.05 and 0.1 M Ca^{2+} solutions increased the activity of InuA by 30 and 10%, respectively).

Acetate, citrate, nitrate, sulfate, phosphate, and chloride ions were tested at concentrations up to 0.25 M

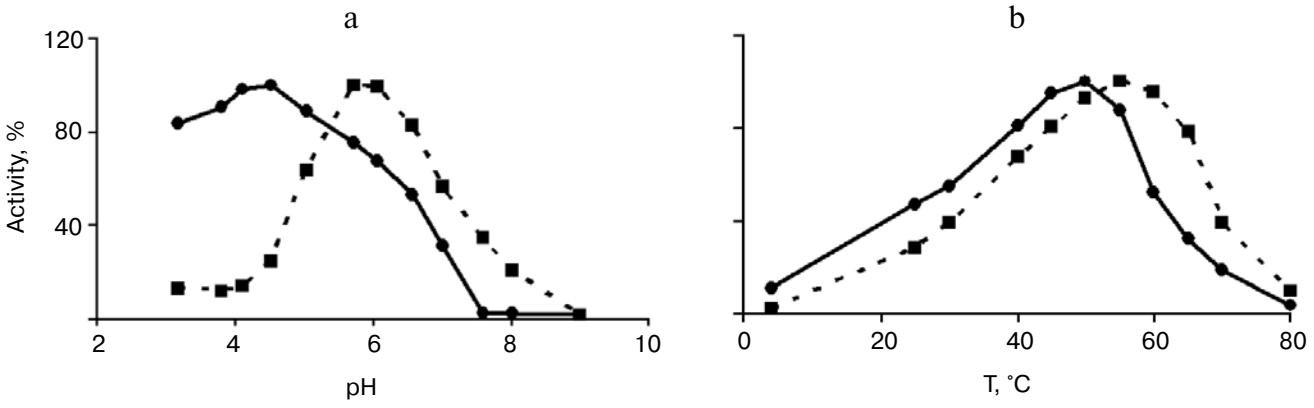

Fig. 7. Dependence of Inu1 (solid curves) and InuA (dashed curves) activity on pH at 50°C (a) and on temperature at pH 5.0 (b) determined by hydrolysis of topinambour inulin.

in experiments on the effect of acid anions on the activity of exo- and endoinulinases to hydrolyze inulin (Table 2). Both enzymes were insensitive to CH_3COO^-, Cl^-, and NO_3^- ions. Sulfate and phosphate ions had no effect on the activity of Inu1, but displayed a significant inhibitory effect on InuA activity. Specifically, endoinulinase activity decreased by 30% in the presence of 0.05 M SO_4^{2-}, by 40% in the presence of 0.10-0.25 M SO_4^{2-}, and 0.05, 0.1, and 0.25 M PO_4^{3-} inactivated enzyme by 40, 60, and 100%, respectively. Citrate ions (0.02 M solution) completely inhibited the catalytic activity of both inulinases.

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