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Isolation and Properties of Major Components of *Penicillium canescens* Extracellular Enzyme Complex

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Abstract—The composition of the enzyme complex secreted by *Penicillium canescens* was investigated. A scheme for purification of the main components of the complex by chromatofocusing on a Mono P column was developed. It was found that along with β-galactosidase, the major components of the complex were endo-β-1,4-xylanase (31 kD, p*I* 8.2-9.3), α-L-arabinofuranosidase (60 kD, p*I* 7.6), arabinoxylan-arabinofuranohydrolase (70 kD, p*I* 3.8-4.0), and endo-β-1,3/1,4-glucanase (40 kD, p*I* 4.4). The substrate specificity, pH and temperature activity optima, adsorbability, thermal stability, and ability for synergic interaction of the isolated enzymes were studied.

Key words: *Penicillium canescens*, endo-β-1,4-xylanase, α-L-arabinofuranosidase, arabinoxylan-arabinofuranohydrolase, endo-β-1,3/1,4-glucanase, chromatofocusing

The micellar fungus *Penicillium canescens* (F-178, VKPM collection, Russia) is a natural soil isolate [1]. This strain was isolated as a producer of β-galactosidase (β-Gal)—an extracellular enzyme that is "major" in protein content in the complex (30% of all secreted protein). In the former Soviet Union this strain was suggested for the industrial production of β-Gal without any additional tests [2]. β-Gal productivity of the natural strain attained 50 U/ml of culture liquid. *P. canescens* β-Gal is a monomeric glycoprotein (7.5% carbohydrate) with molecular mass 120 kD, p*I* 6.7, pH optimum 4.5, and temperature optimum at 55°C [3, 4]. This enzyme is of particular interest because along with hydrolase activity it also possesses β-1,6-trans-galactosylating activity and produces a set of very tasty oligosaccharides from whey lactose [5].

Our studies demonstrated that for *P. canescens*, the rates of culture growth and biosynthesis of extracellular enzymes are rather high (fermentation ends after 96-100 h), the fermentation medium is simple in composition and not expensive, and the fermentation process can be easily scaled. *P. canescens* secrets only trace amounts of proteases, and this decreases the possibility of proteolysis of the proteins formed. All the above-mentioned features make the *P. canescens* strain economically and technologically favorable for production of extracellular enzymes.

However, it should be mentioned that although *P. canescens* has been used in biotechnology for a long time, the composition and properties of the components of enzyme complex secreted by this strain are studied insufficiently. Along with β-Gal mentioned above, only a "minor" component—α-galactosidase—was characterized; the latter is a glycoprotein (5.2% carbohydrates) with molecular mass 60 kD, p*I* 6.7, pH optimum 4.2-4.5, and temperature optimum 45-50°C [6].

The goal of this work was to isolate the main ("major") components of *P. canescens* complex and to study their physicochemical and biochemical properties, substrate specificity, and mechanisms of their action on the polymeric substrates.

Abbreviations: β-Gal) β-galactosidase; CMC) sodium salt of carboxymethylcellulose; PGU) sodium salt of polygalacturonic acid; *p*-NP) *p*-nitrophenyl; MCC) microcrystalline cellulose; ROD-CMC) CMC stained with Reactive Orange GT; RBB-CMC) CMC stained with Remazol Brilliant BlueR.

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MATERIALS AND METHODS

Enzyme preparation. In this study we used ultrafiltrate of culture liquid of *P. canescens* wild strain PCA 10 obtained at the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences (Pushchino, Russia).

Substrates. To determine enzyme activities, the following substrates were used: sodium salt of carboxymethylcellulose (CMC) of medium viscosity, β-glucan from barley, laminarin, curdlan, lichenan from *Cetraria islandica*, glucuronoxylan from birch wood and beech wood, arabinoxylan from oat, xyloglucan from tamarind, sodium salt of polygalacturonic acid (PGU), arabinogalactan from larch wood, raffinose, stachyose, and *p*-nitrophenyl-glycosides (*p*-NP-glycosides: α-L-arabinopyranoside, α-L-arabinofuranoside, α-D-galactopyranoside, β-D-galactopyranoside, α-D-glucopyranoside, β-D-glucopyranoside, α-D-xylopyranoside, β-D-xylopyranoside, β-D-lactopyranoside, α-D-mannopyranoside, β-D-mannopyranoside, β-D-cellobioside, glucuranoside) from Sigma (USA); arabinoxylan from wheat, arabinan from sugar beet, and galactan from potato from Megazyme (Australia); partly depolymerized soluble galactomannan from acacia kindly given by Dr. V. D. Scherbukhin; microcrystalline cellulose (MCC) from Serva (Germany); soluble substrates ROD-CMC (CMC stained with Reactive Orange, GT from Fermtech (Russia)) and RBB-CMC (CMC stained with Remazol Brilliant Blue R from Megazyme). Chromatographic paper No. 1 was from Whatman (England).

Estimation of enzyme activity. Enzyme activity against MCC and unstained soluble polysaccharide substrates were assayed via the initial rates of formation of reducing sugars (RS) by the modified Somogyi–Nelson procedure [7].

Activity against unstained soluble polysaccharide substrates. Preliminarily heated (5 min, 50°C) 160 μl of 0.625% solution of polysaccharide substrate in 0.1 M acetate buffer, pH 5.0, and 40 μl of analyzed enzyme solution were mixed in a thermostatted test tube 2 ml in volume. The reaction mixture was incubated for 10 min (5 min in case of CMC) at 50°C; the reaction was stopped by addition of 200 μl of Somogyi reagent. The tightly stoppered test tube was kept in a boiling water bath for 40 min, then the reaction mixture was cooled to room temperature and 200 μl of Nelson reagent was added. The solution was carefully mixed, incubated for 10 min at room temperature, and after adding 400 μl of acetone and 1 ml of water the mixture was shaken and centrifuged at 13,000 rpm for 1 min. Then optical density was measured at 610 nm and the enzyme activity was calculated, accounting for the contributions of the substrate, enzyme, and acetate buffer solutions determined in blind experiments. Enzyme dilution was adjusted so that the optical density was from 0.5 to 0.9 unit.

Activity against MCC. Preliminarily heated (5 min, 40°C) 200 μl of 1% MCC suspension in 0.1 M acetate buffer, pH 5.0, 120 μl of 0.1 M acetate buffer, pH 5.0, and 80 μl of analyzed enzyme were mixed in a thermostatted test tube. The reaction mixture was incubated with stirring for 30 min at 40°C, centrifuged at 8000 rpm for 30 sec, 200 μl of the supernatant was quickly sampled and placed in a test tube with 200 μl of Somogyi reagent. Then the procedure was the same as described above for the unstained soluble polysaccharide substrates. Enzyme dilution was adjusted so that the resulting optical density at 610 nm was from 0.2 to 0.4 unit.

Enzyme activities against other substrates were determined according to standard procedures [7]. Endoglucanase activity (CMC of medium viscosity as the substrate) was determined viscosimetrically at pH 5.0 and 40°C and activity against filter paper (FP) at pH 5.0 and 50°C. Enzyme activities against stained polysaccharides (ROD-CMC and RBB-CMC) and synthetic low-molecular-weight substrates (*p*-NP-glycosides) were assayed via the initial rates of formation of the stained reaction products at pH 5.0 and 40°C.

Specific enzyme activities are expressed in international units per mg protein, U/mg (one activity unit corresponds to the amount of the enzyme hydrolyzing 1 μmol of glycoside bonds of the substrate per minute).

Chromatography of the products of hydrolysis of polysaccharide substrates. The products of enzymatic hydrolysis of the polysaccharide substrates were identified using a Chromatography Workstation 700 liquid chromatograph from Bio-Rad (USA). Saccharides were detected using a differential refractometer Knauer (Germany).

Change in molecular-mass distribution (MMD) of the polysaccharide substrates in the course of hydrolysis was monitored by high-pressure gel penetration chromatography (GPC) [8] on a Bio-Gel TSK 30XL column (6 μm, 250 × 4 mm) from Bio-Rad. Acetate buffer (0.1 M, pH 5.0) was used as the eluent; the flow rate was 1 ml/min.

Low-molecular-weight products of hydrolysis of the polysaccharide substrates were identified by HPLC on a Diasorb-130-NH_2 column with grafted amino phase (7 μm, 250 × 4.6 mm) from BioChemMak (Russia). A mixture of acetonitrile–water at the ratio of 7 : 3 (or 8 : 2 for the better resolution of xylose, arabinose, and xylobiose peaks) was used as the eluent, the flow rate being 1 ml/min.

For calibration plots, the following reagents were used as the standards: for high-pressure GPC, dextrans with molecular masses 20-250 kD from Pharmacia (Sweden) and maltopentaose, maltotetraose, and maltotriose from Sigma; for HPLC, D-xylobiose, D-xylose, D-cellobiose, and α-L-arabinose from Merck (Germany), D-glucose from Sigma, D-galactose from Reakhim (Russia), and all above-mentioned maltooligosaccharides.

Isolation and purification of components of the enzyme complex [9, 10]. Enzyme preparation was precipitated with ammonium sulfate (80% saturation at 0°C). Precipitate formed during 12 h (4°C) was centrifuged at 11,000 rpm for 30 min at 4°C. Desalting and replacement of buffers were performed by low-pressure GPC on the columns with Biogel P4 from Bio-Rad using an Econo System liquid chromatograph from Bio-Rad with flow rate 1 ml/min.

To isolate the main components of the complex, a FPLC liquid chromatograph from Pharmacia was used. During isolation, the protein concentration was determined spectrophotometrically at 280 nm. Chromatofocusing (ion-exchange chromatography at a low ionic strength with pH gradient elution) was performed on a Mono P column (5 × 200 mm, gel volume 4 ml) from Pharmacia equilibrated with the initial buffer (0.075 M Tris/AcOH, pH 8.3). The pH gradient was created by sequential passage of polybuffers through the column: PB 96 with pH 6.0, PB 74 with pH 4.0, and PB 74 with pH 3.0, the flow rate 0.5-0.6 ml/min.

For fine protein purification, a second chromatofocusing on a Mono P column with elution of the bound protein in a narrow pH gradient (8-6, 6-4, and so on) was performed. Various combinations of the initial buffers (Tris/AcOH, pH 9.3; 0.025 M imidazole/HCl, pH 7.4; 0.025 M bis-Tris/HCl, pH 6.3) and polybuffers (PB 96, pH 6.0 and 7.0; PB 74, pH 6.0, 5.0, 4.0, and 3.0) at flow rate 0.6 ml/min were used.

The protein concentration in the samples was determined according to Lowry using BSA as the standard [11] or spectrophotometrically at 280 nm.

Determination of biochemical characteristics of individual enzymes [9, 10]. To prepare the plates (70 × 80 × 0.75 mm) with polyacrylamide gel for electrophoresis under the denaturing conditions (SDS-PAGE) with concentrating and separating gels (4 and 12% polyacrylamide gel, respectively) and also for isoelectrofocusing (IEF) in 4% polyacrylamide gel (125 × 65 × 0.75 mm), the individual reagents and kits from Reanal (Hungary), Sigma, and Bio-Rad were used. Analytical IEF of individual proteins was performed using a Model III Cell and SDS-PAGE with a Mini Protean II from Bio-Rad according to the standard procedures. The protein bands in the gels were stained with Coomassie Brilliant Blue R-250 from Ferak (Germany) in 25% TCA or 0.1% $AgNO_3$ from Poch (Poland). Before electrophoresis the studied enzyme solutions were treated with 1% SDS and 5% β-mercaptoethanol for 15 min at 100°C.

Molecular Weight Markers protein mixtures from Sigma were used as the standards for SDS-PAGE.

To determine the content of carbohydrates in proteins according to Dubois [7], analytically pure phenol and H_2SO_4 from Reakhim and D-mannose from Sigma were used.

Determination of kinetic characteristics and physicochemical properties of the homogeneous enzymes. To study the thermal stability of the homogeneous enzymes and the pH dependence of their activity, a universal buffer was used [11]. Experiments on the degree of complete hydrolysis of polysaccharide substrates [12], the coefficient of synergism K_s [7], adsorbability of the enzymes on insoluble substrates (MCC and glucuronoxylan from beech wood) [7], and temperature dependence of enzyme activity were performed using 0.1 M acetate buffer. The degree of complete hydrolysis of polysaccharide substrates (initial substrate concentration 5 g/liter) and the effect of synergism (substrate concentrations 0.5-5 g/liter) were studied at pH 5.0 and 50°C, and adsorbability (substrate concentrations 1-30 g/liter) at pH 4.5 and cooling in an ice bath.

RESULTS AND DISCUSSION

Isolation of the main components of enzyme complex of *P. canescens*. The main scheme allowing fast and efficient isolation of individual homogeneous components of *P. canescens* enzyme complex in amounts sufficient for their detailed study included three main steps: preliminary purification of the enzyme complex from non-protein impurities, rough fractionating of the enzyme complex by chromatofocusing in a wide range of pH (8-3), and fine purification including additional fractionating of protein fractions sampled at the preceding step and containing the main part of the studied enzyme or enzymes.

P. canescens preparation preliminarily purified from non-protein impurities (insoluble substances, carbohydrates, pigments, etc.) was chromatofocused on Mono P.

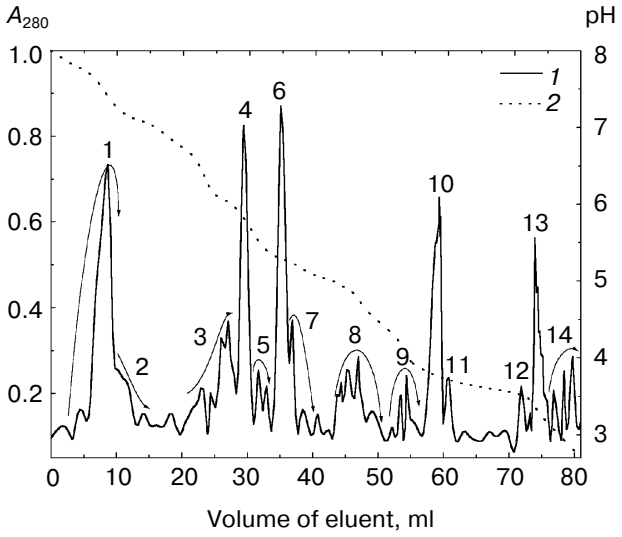

Fig. 1. Chromatofocusing on Mono P of *P. canescens* enzyme complex: *1*) protein, A_{280}; *2*) pH at the exit from the column; 1-14) fraction numbers.

As can be seen on the chromatogram (Fig. 1), the main part of protein was eluted in the composition of fractions 1, 4, 6, 8, 10, and 13, fractions 1, 4, 6, 8, and 10 containing major proteins. Analyzing activities of the fractions (more than 30 substrates of various composition and structure were used for this), it was found that fraction 1 is specific for various xylanes, hydrolyzes *p*-NP-cellobioside, and possesses negligible activity against polymeric cellulose substrates; fraction 6 actively hydrolyzes laminarin, β-glucan, and lichenan; fractions 4 and 10 are most active against *p*-NP-α-L-arabinofuranoside and polymeric arabinoside-containing substrates. Fraction 8 was specific for such substrates as *p*-NP-β-galactopyranoside, raffinose, and stachyose, that is, those containing β-Gal, and was not subjected to further purification.

For isolation of individual enzymes of *P. canescens* complex, fractions 1, 4, 6, and 10 were chosen. Chromatofocusing appeared to be the optimal method of separation providing the maximal yields of highly homogeneous proteins with minimal activity loss. Each fraction was twice purified on Mono P, pH gradients being as follows: 8.0-5.6 for fraction 1, 6.0-3.8 for fraction 4, 5.6-3.6 for fraction 6, and 4.6-2.7 for fraction 10 (chromatograms of the final step of purification are presented in Fig. 2). Thus, four homogeneous major components were isolated from the enzyme complex of *P. canescens* PCA 10: proteins with molecular masses 31 kD (p*I* 8.8-9.3), 60 kD (p*I* 7.6), 40 kD (p*I* 4.4), and 70 kD (p*I* 3.8-4.0) (Fig. 3).

Substrate specificity and classification of isolated enzymes. For accurate classification of isolated enzymes, their substrate specificity and type of depolymerization of polysaccharide substrates (endo- and exo-) were analyzed. Data on the qualitative composition of the final low-molecular-weight products of hydrolysis of polysaccharide structures, data on change in MMD of polysaccharides, and data on the total RS yield at complete hydrolysis of these substrates were used to clarify the type of enzymatic action.

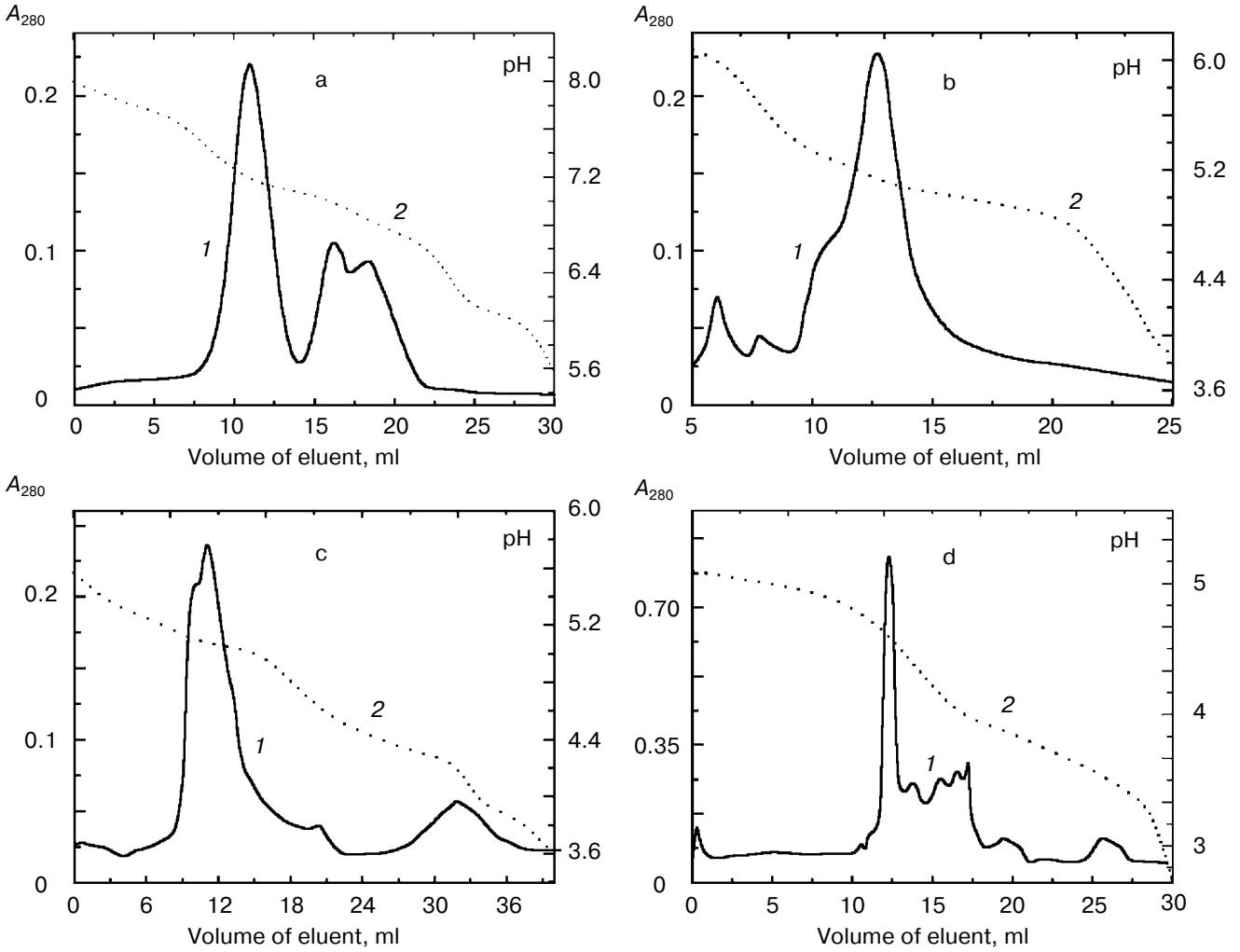

Fig. 2. Final step of isolation of the main components of *P. canescens* enzyme complex by chromatofocusing on Mono P from fractions 1 (a), 4 (b), 6 (c), and 10 (d): *1*) protein, A_{280}; *2*) pH at the exit from the column.

Enzyme with molecular mass 31 kD showed the highest specific activities against glucurono- and arabinoxylan (28.1 and 23.9 U/mg, respectively) (Table 1) but did not hydrolyze insoluble cellulose (MCC, FP). However, it appeared that this enzyme is able to hydrolyze soluble derivatives of cellulose (CMC, RBB-CMC, and ROD-CMC) and β-1,4-glucans (β-glucan from barley and lichenan). This suggests that the enzyme combines the properties of xylanases and cellulases. The absence of activity against low-molecular-weight soluble substrates (excluding *p*-NP-cellobioside) indicates that the action of this enzyme is of endodepolymerase type. This is proved by data on change in MMD of glucurono- and arabinoxylan. The average molecular mass of the substrates significantly decreases at the initial step of hydrolysis, 10-20 min (Fig. 4a), whereas the relative content of low-molecular-weight products in the reaction mixture was negligible (e.g., after 1 h hydrolysis the area of a peak corresponding to the low-molecular-weight products was only 8-10% of the total area of the chromatographic profile). The degree of conversion of glucurono- and arabinoxylan was 35-40% and the final yield of oligosaccharides was markedly higher than the yield of monomer (xylobiose > xylotriose > xylose). These data indicate that the isolated enzyme is an endo-β-1,4-xylanase with the ordered mechanism of action. Ability to hydrolyze *p*-NP-cellobioside is a specific feature of this enzyme typical for xylanases of family 10 of glycoside hydrolases. The enzyme was assigned the name Xyl-31.

The enzyme with molecular mass 60 kD actively hydrolyzed *p*-NP-α-L-arabinofuranoside (8.1 U/mg, Table 1), was specific for furanose configuration of the carbohydrate ring, and did not hydrolyze *p*-NP-α-L-arabinopyranoside. The enzyme also exhibited significant activity against all polymeric arabinose-containing substrates (Table 1). Activity against arabinogalactan was lower; arabinose units of this polymeric molecule mainly have pyranose configuration. It is important that change in the average molecular mass of arabinan and arabinoxylan by the action of the enzyme on these substrates was negligible (Fig. 4c) with the rapid growth of the relative content of low-molecular-weight products in the mixture (after 1 h hydrolysis the area of a peak corresponding to the low-molecular-weight products was 40-50% of the total area of the chromatographic profile). The degree of completeness of hydrolysis of arabinoxylan and arabinan was not large (15 and 20%, respectively), and arabinose was the only low-molecular-weight product in both cases. The data allowed classification of this enzyme as an α-L-arabinofuranosidase; it was assigned the name AF-60.

The enzyme with molecular mass 40 kD exhibited the highest specific activity against laminarin (8.8 U/mg, which is three times higher than the specific activity against CMC, Table 1). Along with laminarin, the enzyme hydrolyzed other soluble polymeric glucans having β-1,3-glucoside bonds: β-glucan from barley, lichenan, and curdlan. Activity against insoluble cellulose substrates, FP and MCC, and also low-molecular-weight *p*-NP-derivatives of mono- and disaccharides was not observed. On hydrolysis of laminarin and β-glucan the average molecular mass of the substrates drastically decreased at the initial stage of hydrolysis, 10-20 min (Fig. 4b), whereas the relative content of low-molecular-weight products of hydrolysis was negligible (after 1 h hydrolysis the area of a peak corresponding to the low-molecular-weight products was only 8-12% of the total area of the chromatographic profile). The degree of laminarin and β-glucan conversion was 40 and 50%, respectively; glucose was the main final product of hydrolysis. The data allowed classification of this enzyme as an endo-

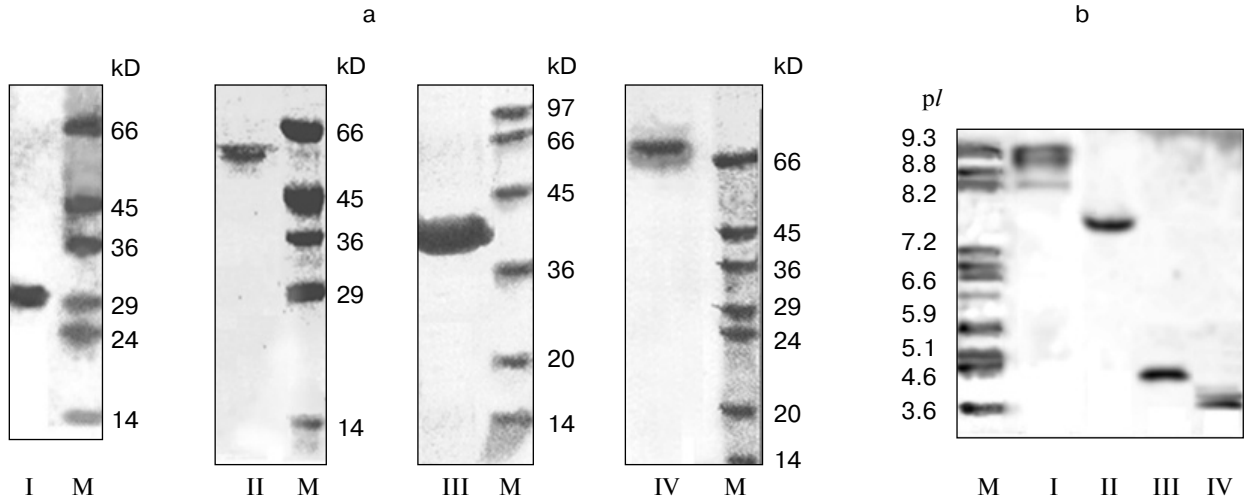

Fig. 3. Biochemical parameters of the major components of *P. canescens* enzyme complex obtained by SDS-PAGE (a) and IEF (b). Enzymes from: I) fraction 1; II) fraction 4; III) fraction 6; IV) fraction 10. M, standard markers.

Table 1. Specific activities (U/mg) of the main components of enzyme complex of *P. canescens* (pH 5.0, 50°C)

Substrate	Enzyme			
	31 kD (Xyl-31)	60 kD (AF-60)	40 kD (Lam-40)	70 kD (AF-70)
CMC	1.2	0.1	**2.6**	0.07
RBB-CMC*	**3.1**	0.2	**5.8**	0
ROD-CMC*	0.8	0.04	0.8	0.01
FP	0.01	0	0	0
MCC	0	0	0	0
Laminarin	0.2	0.1	**8.8**	0.1
β-Glucan	**2.2**	0.3	**5.0**	0.1
Lichenan	0.8	0.06	**3.4**	0
Curdlan	0.05	0.04	0.6	0.01
Xyloglucan	**6.3**	0.9	1.1	**5.6**
Glucuronoxylan	**28.1**	**2.4**	0.3	0.2
Arabinoxylan	**23.9**	**7.4**	0.3	**11.6**
Arabinan	0.4	**6.9**	0.3	**5.6**
Arabinogalactan	0.6	1.0	0	1.9
Galactomannan	0.8	0.4	0.2	1.6
Galactan	0	0	0	1.1
PGU	1.1	0.5	0.3	0.3
p-NP-β-D-cellobioside*	**5.3**	0	0	0
p-NP-β-D-lactopyranoside*	0	0	0	0
p-NP-α-D-glucopyranoside*	0	0	0	0
p-NP-β-D-glucopyranoside*	0	0	0	0
p-NP-α-D-galactopyranoside*	0.03	0.02	0.01	1.0
p-NP-β-D-galactopyranoside*	0.02	0	0.01	**7.1**
p-NP-α-D-mannopyranoside*	0.01	0.02	0.01	0
p-NP-β-D-mannopyranoside*	0	0.02	0	0
p-NP-α-D-xylopyranoside*	0.02	0	0.01	0
p-NP-β-D-xylopyranoside*	0	0	0	0.1
p-NP-α-L-arabinopyranoside*	0	0	0.02	0.7
p-NP-α-L-arabinofuranoside*	0.2	**8.1**	0.04	**12.5**
p-NP-glucuranoside*	0	0	0	0

* Activity against these substrates was estimated at 40°C.

β-1,3/1,4-glucanase (β-glucanase) acting via the ordered mechanism; it was assigned the name Lam-40.

The enzyme with molecular mass 70 kD exhibited substrate specificity to some extent similar to that of AF-60 described above (Table 1). Analogously to AF-60, this enzyme was not able to change significantly molecular masses of arabinoside-containing polymeric substrates (Fig. 4d), and on interaction with arabinan, arabinose was the only low-molecular-weight product. The data indicate that the 70-kD enzyme is an α-L-arabinofuranosidase. However, analysis of the low-molecular-weight products of enzymatic hydrolysis of arabinoxylan showed that along with arabinose, xylobiose and several oligosaccharides with unknown structure are formed, the final degree of conversion of arabinoxylan being 1.5 times higher than that for AF-60 (although on hydrolysis of arabinan an opposite situation was observed, Table 2). Probably, on hydrolysis of arabinoxylan the 70-kD enzyme cleaved not only side arabinose residues but also hydrolyzed xyloside bonds in the main polymeric chain via exodepolymerizing mechanisms with formation of xylobiose. The exo-

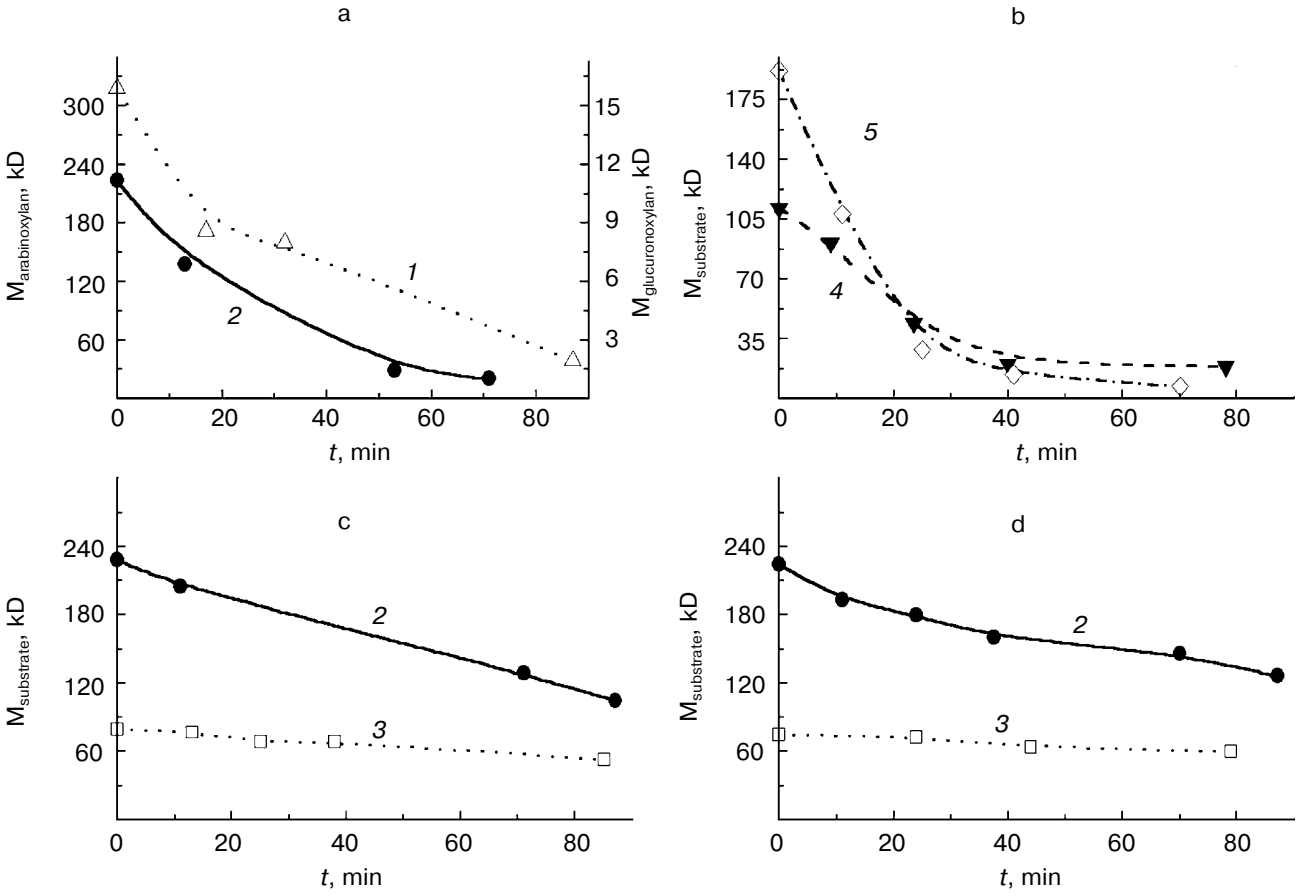

Fig. 4. Average molecular mass (M) of products of polysaccharide hydrolysis by the action of Xyl-31 (a), Lam-40 (b), AF-60 (c), and AF-70 (d) determined by high-pressure GPC: *1*) glucuronoxylan; *2*) arabinoxylan; *3*) arabinan; *4*) β-glucan; *5*) laminarin.

mechanism of depolymerization was proved by the change in MMD of arabinoxylan: at the first stage of hydrolysis the relative content of low-molecular-weight products grew rapidly (after 1 h hydrolysis the area of a peak corresponding to the low-molecular-weight products was only 8-12% of the total area of chromatographic profile) with a small decrease in the average molecular mass of the substrate. Thus, we conclude that the 70-kD enzyme is an arabinoxylan-arabinofuranohydrolase [13-15]; it was assigned the name AF-70. A specific feature of AF-70 (compared with AF-60) is its wider specificity manifesting itself as ability to hydrolyze low-molecular-weight and polymeric galactoside-containing substrates, e.g., galactan, xyloglycan, and *p*-NP-β-D-xylopyranoside.

Adsorbability. Xyl-31, Lam-40, AF-60, and AF-70 were poorly adsorbed on MCC as well as on insoluble glucuronoxylan from beech wood: the values of K_d, the distribution coefficient, did not exceed 0.03 liter/g (Table 2). This indirectly indicates that the adsorption domain is absent from the molecular structures of the studied enzymes. The K_d values typical of highly adsorbed enzymes containing adsorption domains tens and even hundreds of times exceed the values obtained by us.

Temperature and pH dependence of enzyme activity. As shown in Fig. 5, pH dependences of activity of the enzymes under investigation have maxima in the pH range 4-6. AF-60 appeared to be the most "acidic" (using *p*-NP-α-L-arabinofuranoside as the substrate): pH optimum 4.7. For AF-70, the pH optimum is closer to the neutral value: 5.5 (with the same substrate), but the form and character of pH dependences for both substrates coincide, and both AF-60 and AF-70 retain 40% activity at pH 2.0. On hydrolysis of arabinoxylan, the activity optimum of AF-70 is observed at pH 4.8, although the enzyme retains only 20% maximal activity at pH 2.0. Lam-40 also retains a significant part of its activity in highly acidic medium (~40% at pH 2.3). The pH profile of this enzyme is "spade-like": its activity drastically decreases when pH slightly deviates from the optimal value 4.9. For Xyl-31, the pH optimum (5.9) is close to neutral values, and at pH < 4.0

Table 2. Properties of the main components of *P. canescens* enzyme complex

Parameters	Enzyme			
	Xyl-31	AF-60	Lam-40	AF-70
Molecular mass, kD	31	60	40	70
p*I*	8.2-9.3*	7.6	4.4	3.8-4.0*
Hydrolyzed bonds	Internal β-1.4-$(Xyl)_n$	Terminal α-L-Ara f	Internal β-1,3/1,4-$(Glc)_n$	Terminal α-L-Ara f, Internal β-1.4-$(Xyl)_n$
Mechanism of action	"Endo", ordered	"Exo"	"Endo", ordered	"Exo"
Degree of completeness of hydrolysis, %	35 (arabinoxylan)	15 (arabinoxylan)	40 (β-glucan)	26 (arabinoxylan)
	40 (glucuronoxylan)	20 (arabinan)	50 (laminaran)	11 (arabinan)
K_d, liter/g**				
MCC	0.01	0.01	0.02	0.02
Xylan from beech wood	0.03	0.02	0.01	0.03

* According to IEF data, molecules of these enzymes have several isoforms.
** The distribution coefficient of adsorption on insoluble substrate.

and pH > 7.5 the enzyme loses up to 80% of its initial activity.

As shown in Fig. 6, for the activities of Xyl-31, Lam-40, AF-60, and AF-70 the temperature maxima vary from 55°C (Xyl-31) to 70°C (AF-60). AF-60 and AF-70 appeared to be the most "thermophilic": they exhibited 50% maximal activity at 75°C. For Lam-40, the temperature optimum was in the range 60-65°C, the enzyme exhibiting 30% activity at low (25°C) as well as at high (90°C) temperatures. Xyl-31 exhibited maximal activity within a relatively narrow temperature range 55-61°C.

Stability of the enzymes was studied at pH 4-6 (the values near pH optima) and 40-60°C. The results are presented in Table 3. Lam-40 appeared to be the least stable:

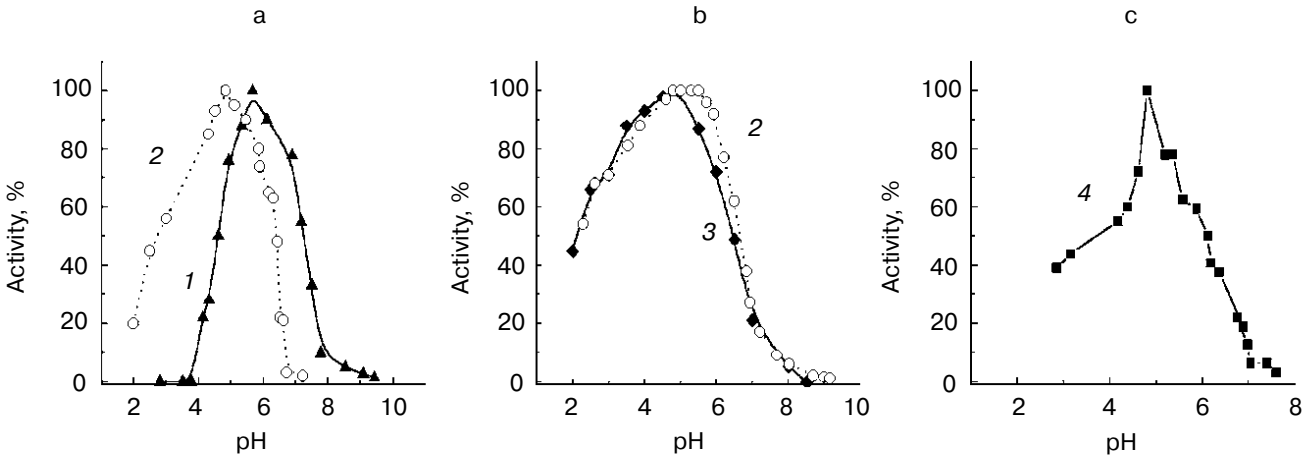

Fig. 5. pH dependence of enzyme activities estimated on hydrolysis of specific substrates: glucuronoxylan from birch wood (a), *p*-NP-α-L-arabinofuranoside (b) and laminarin (c). *1*) Xyl-31; *2*) AF-70; *3*) AF-60; *4*) Lam-40. Conditions: reaction time 10 min, 50°C.

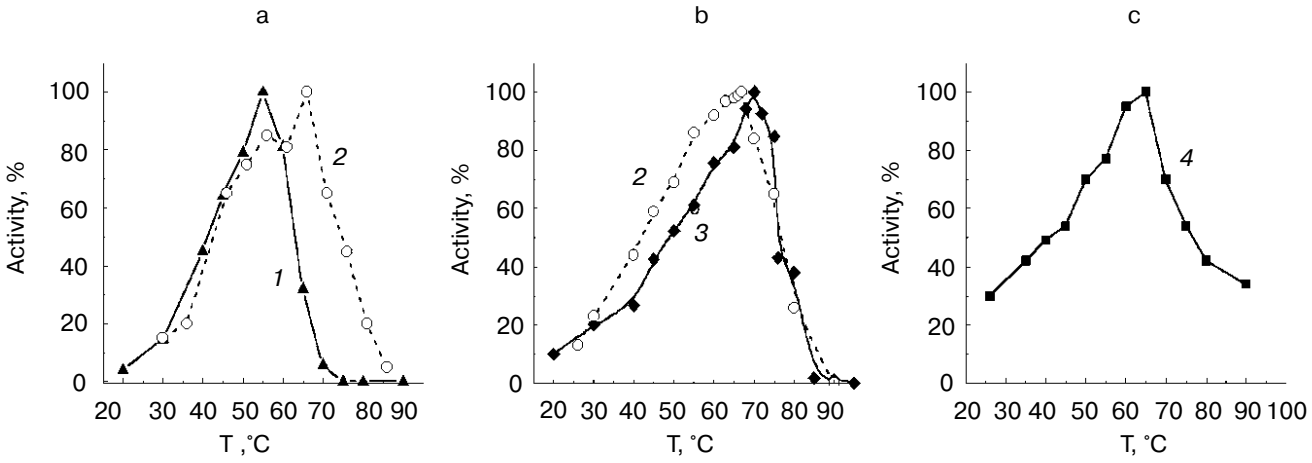

Fig. 6. Temperature dependence of enzyme activities estimated on hydrolysis of specific substrates: glucuronoxylan from birch wood (a), *p*-NP-α-L-arabinofuranoside (b), and laminarin (c). *1*) Xyl-31; *2*) AF-70; *3*) AF-60; *4*) Lam-40. Conditions: reaction time 10 min, pH 5.0.

it lost from 50 to 80% of initial activity during 3 h incubation at 40°C (depending on pH optima) and was completely inactivated at 50 and 60°C. Xyl-31 appeared to be somewhat more stable: it lost from 40% (pH 5.0) to 70% (pH 4.0) of its initial activity during 4 h incubation at 35°C; it lost 80% of its activity during the same period of time at 45°C, and at pH 4.0 and 6.0 the enzyme was completely inactivated after 1 h at 55°C. AF-70 was more resistant to prolonged heating: it retained from 60% (pH 6.0) to 80% (pH 4.0) activity during 3 h incubation at 40°C; the enzyme retained 50-60% of its activity (pH 4.0 and 5.0) at 50°C and was completely inactivated after ~1 h incubation at 60°C. AF-60 appeared to be the most stable: during 3 h incubation it retained 75-80% initial activity at 40 and 50°C and 50% activity at 60°C.

Half-inactivation time for each enzyme was calculated (or approximately evaluated) from kinetic curves of thermal inactivation of Xyl-31, Lam-40, AF-60, and AF-70 at various temperatures and pH values. The results are presented in Table 3.

Synergetic interactions between the enzymes. Enzyme complexes of carbohydrases produced by

Table 3. Stability of the main components of *P. canescens* enzyme complex

Incubation conditions		Half-inactivation time, min*			
T, °C	pH	Xyl-31**	AF-60	Lam-40	AF-70
40	4.0	180	>>180	90	>>180
	5.0	>180	>>180	60	>180
	6.0	>180	>>180	55	>180
50	4.0	15	>>180	130	>>180
	5.0	180	>>180	110	>180
	6.0	130	>>180	100	15
60	4.0	1	>180	120	25
	5.0	5	180	100	15
	6.0	2	90	10	10

* Estimated via the loss of enzyme activity on hydrolysis of glucuronoxylan from birch wood (Xyl-31), *p*-NP-α-L-arabinofuranoside (AF-60 and AF-70) and laminarin (Lam-40).

** For xylanase, temperature was 35, 45, and 55°C, respectively.

microorganisms are systems in which efficiency of joined (simultaneous) action of enzymes is higher than the effect of their individual actions, that is, synergism is observed (synergism between the enzymes hydrolyzing hemicellulose was described in [17-20]).

We found that the yield of RS at deep (complete) hydrolysis of arabinoxylan by *P. canescens* enzyme complex is higher than that by the action of its individual enzymes – the degree of complete hydrolysis of arabinoxylan by the initial enzyme preparation was 85-90%, whereas the degree of hydrolysis of arabinoxylan by the action of Xyl-31, AF-60, and AF-70 individually varied from 15 to 35% (Table 2). The degree of complete hydrolysis of arabinoxylan by simultaneous action of Xyl-31, AF-60, and AF-70 was ~80%. The data suggest that on hydrolysis of arabinoxylan the enzymes of *P. canescens* act synergistically.

Thus, studying the components of the enzyme complex of the *P. canescens* wild strain it was found that along with β-galactosidase (120 kD, p*I* 6.7), endo-β-1,4-xylanase (31 kD, p*I* 8.2-9.3, tenth family of glycosylhydrolases), α-L-arabinofuranosidase (60 kD, p*I* 7.6), arabinoxylan-arabinofuranohydrolase (70 kD, p*I* 4.4), and endo-β-1,3/1,4-glucanase (40 kD, p*I* 3.8-4.0) are also major enzymes of this complex.

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