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Isolation and Properties of Pectinases from the Fungus *Aspergillus japonicus*

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Abstract—Using anion-exchange chromatography on different carriers and phenyl-Sepharose hydrophobic chromatography, five pectolytic enzymes were isolated from the culture liquid of a mutant strain of *Aspergillus japonicus*: two endo-polygalacturonases (I and II, 38 and 65 kD, p*I* 5.6 and 3.3), pectin lyase (50 kD, p*I* 3.8), and two pectinesterases (I and II) with similar molecular weights (46 and 47 kD) and the same p*I* (3.8). The pectinesterases apparently represent two isoforms of the same enzyme. All purified enzymes were homogenous according to SDS-PAGE and polyacrylamide gel-IEF, except for endo-polygalacturonase II that gave two bands on isoelectric focusing, but one band on electrophoresis. All enzymes had maximal activity in an acid medium (at pH 4.0-5.5). The pectin lyase and pectinesterase were stable at 40-50°C. The thermal stability of both endo-polygalacturonases was much lower (after 3 h of incubation at 30°C, endo-polygalacturonases I and II lost 40 and 10% of the activity, respectively). The activity of endo-polygalacturonases I and II towards polygalacturonic acid strongly depended on NaCl concentration (optimal concentration of the salt was 0.1-0.2 M); the enzymes were also capable of reducing the viscosity of pectin solution, but rather slowly. The pectin lyase had no activity towards polygalacturonic acid. The activity of the pectin lyase increased with increasing degree of methylation of pectins. Both endo-polygalacturonases demonstrated synergism with the pectinesterase during the hydrolysis of highly methylated pectin. On the contrary, in the mixture of pectin lyase and pectinesterase an antagonism between the two enzymes was observed.

Key words: pectins, pectinase, polygalacturonase, pectin lyase, pectinesterase, chromatography

Enzymes involved in degradation of pectins are widespread in nature and can be found in many plants, bacteria, and fungi [1-3]. The most important enzymes of a pectinase complex are polygalacturonase (EC 3.2.1.15), pectin lyase (EC 4.2.2.10), pectate lyase (EC 4.2.2.2), and pectinesterase (EC 3.1.1.11) [1, 2]. Recently pectinases have become more widely used in food, textile, and other branches of industry.

The best known microbial producers of pectinases are different species of *Aspergillus* fungi exhibiting as a rule a wide spectrum of pectinase activities [3, 4]. Enzyme preparations based on *Aspergillus* have been successfully used in practice, being employed for processing of vegetables and fruits, as well as for clarification of juices and wines. Among the commercial pectinolytic enzymes, preparations obtained by the industrial cultivation of different strains of *Aspergillus niger* and *Aspergillus awamori* are the most popular ones [3, 4].

The goal of the present study was to isolate and study the properties of the enzymes of the pectinase complex that is produced by a mutant strain of *Aspergillus japonicus*, a promising producer of hemicellulases and pectinases.

MATERIALS AND METHODS

Enzymes. The enzyme preparation from *A. japonicus* was obtained in the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino). The preparation was an ultrafiltrate of the culture liquid of the fungal culture.

Substrates. Polygalacturonic acid, citrus pectins of methylation degree (MD) of 26, 65, and 89%, pectin containing 9% of methoxyl groups (MD ~ 70%), carboxymethylcellulose, and xylan were from Sigma (USA); xyloglucan, linear and branched arabinans, and galactan were from Megazyme (Australia); galactomannan was from the Laboratory of Carbohydrates (Bach Institute of

Abbreviation: MD) methylation degree.

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Biochemistry, Russian Academy of Sciences); potato starch was from NPO of Starch Products (Russia).

Assay of enzyme activities. The polygalacturonase activity was assayed using the Somogyi–Nelson method [5, 6] by measuring the content of the reducing sugars formed during 10 min of incubation of 0.5% polygalacturonic acid in the presence of the enzyme at 40°C and pH 5.0. The pectin-lyase activity was assayed by measuring the initial rate of the accumulation of Δ-4,5-unsaturated products of pectin degradation [7]. An aliquot (0.1 ml) of the enzyme solution was added into a spectrophotometric cuvette containing 2.9 ml of 0.24% substrate solution in 0.05 M acetate buffer, pH 5.0. The cuvette was thermostatted at 40°C. The kinetics of the accumulation of the unsaturated products of the reaction was recorded at 232 nm. In both cases, the unit of the activity was defined as the amount of the enzyme catalyzing formation of 1 μmol of the product per 1 min of the reaction.

Activity of pectinases was also determined by measuring decrease in the viscosity of a solution of pectin containing 9% of methoxyl groups [8]. The unit of the pectinase activity was defined as the amount of the enzyme providing 50% decrease in the relative viscosity of 0.5% pectin solution for 5 min at pH 5.0 and 40°C.

Pectinesterase activity was determined by measuring the change in the pH value of the medium due to the formation of the free carboxyl groups during the hydrolysis of the methoxyl bonds of pectin (MD ~ 70%) in the presence of pectinesterase. The activity was estimated qualitatively at pH 5.5 using Bromocresol Green (Sigma, USA) as the pH indicator: in the presence of pectinesterase the color of the reaction mixture changed from blue to yellow [9]. To determine the pectinesterase activity quantitatively, a modification of this method [10, 11] was used based on the titration of the released carboxyl groups in pectin using a pH-stat. A pectin solution (0.5% in 0.1 M NaCl, 5 ml) was placed into a cell of the pH-stat (Radiometer, Denmark), and the pH value of the solution was adjusted to 5.0 using an alkali solution. Then 0.1 ml of the enzyme solution was added, and the carboxyl groups released were titrated with 10 mM NaOH at the given pH value. The unit of the pectinesterase activity was defined as the amount of the enzyme that requires spending of 1 μmol of NaOH per 1 min under the conditions of the reaction. While studying the pH dependence of the pectinesterase activity, the titration was performed in the range of pH 3.5-6.0.

The enzymatic activities towards other polysaccharide substrates (cellulase, xylanase, arabinase, galactanase, mannanase, amylase activities) were determined by measuring the initial rates of formation of the reducing sugars by the Somogyi–Nelson method [5, 6]. The unit of the activity was defined as the amount of the enzyme yielding 1 μmol of sugars per 1 min in the presence of the corresponding substrate (0.5%) at pH 5.0 and 50°C.

The protein content in samples was determined by the Lowry method using bovine serum albumin as the standard or by measuring the absorption at 280 nm [12].

Isolation and purification of components of the pectinase complex. The general scheme of the purification is shown in Fig. 1.

To isolate polygalacturonase I, pectinesterases I and II, and pectin lyase, anion-exchange chromatography was employed on a Source 15Q column (1.6 × 5 cm) using an FPLC system (Pharmacia, Sweden). The preparation (an ultrafiltrate of the culture liquid) was desalted on a P2 Acrylex column (Reanal, Hungary) in 20 mM piperazine-HCl buffer, pH 5.5. A sample containing 150 mg of the protein was applied to the Source 15Q column equilibrated with the same buffer. The column was washed with the starting buffer, and then the bound protein was eluted with a NaCl gradient at 5 ml/min (gradient volume, 400 ml). This stage of purification was performed in three batches, and then the fractions exhibiting similar activities were pooled.

The fractions exhibiting pectinase activities were subjected to hydrophobic chromatography on a phenyl-Sepharose column (Phenyl-Sepharose High Performance, 1.6 × 5 cm, Pharmacia). First, dry ammonium sulfate was added to the samples with stirring to achieve the final concentration of 2 M (in the case of the polygalacturonase I fraction, 2.5 M). Then the samples were applied to the column equilibrated with 50 mM sodium-acetate buffer, pH 5.0, containing 2.0 or 2.5 M ammonium sulfate, respectively. The bound proteins were eluted with a 2.0-0 or 2.5-0 M reverse gradient of ammonium sulfate at 3 ml/min (gradient volume, 200 ml).

In the case of polygalacturonase I, two batches of purification on phenyl-Sepharose were performed, loading 39 mg of the protein each time, and the analogous fractions were pooled. The fractions containing homogenous polygalacturonase I were dialyzed in the cold against 20 mM sodium-acetate buffer for 10 h.

The pectinesterase and pectin lyase fractions after the hydrophobic chromatography were subjected to subsequent purification using anion-exchange chromatography on a Mono Q HR 5/5 column (1 ml, Pharmacia). The samples were passed through the P2 Acrylex column equilibrated with 20 mM N-methyl-piperazine-HCl buffer, pH 4.0. Then the samples were applied on the Mono Q column equilibrated with the same buffer, the column was washed with the starting buffer, and then the bound protein was eluted with a 0-0.4 M linear gradient of NaCl at 1 ml/min (gradient volume, 40 ml). In the cases of the pectinesterase I and pectin lyase, the obtained fractions contained the homogenous proteins. The pectinesterase II was purified using again Mono Q HR 5/5 anion-exchange chromatography under the same conditions but at pH 6.0.

To isolate polygalacturonase II, anion-exchange chromatography on a DEAE-Spheron column (2.6 ×

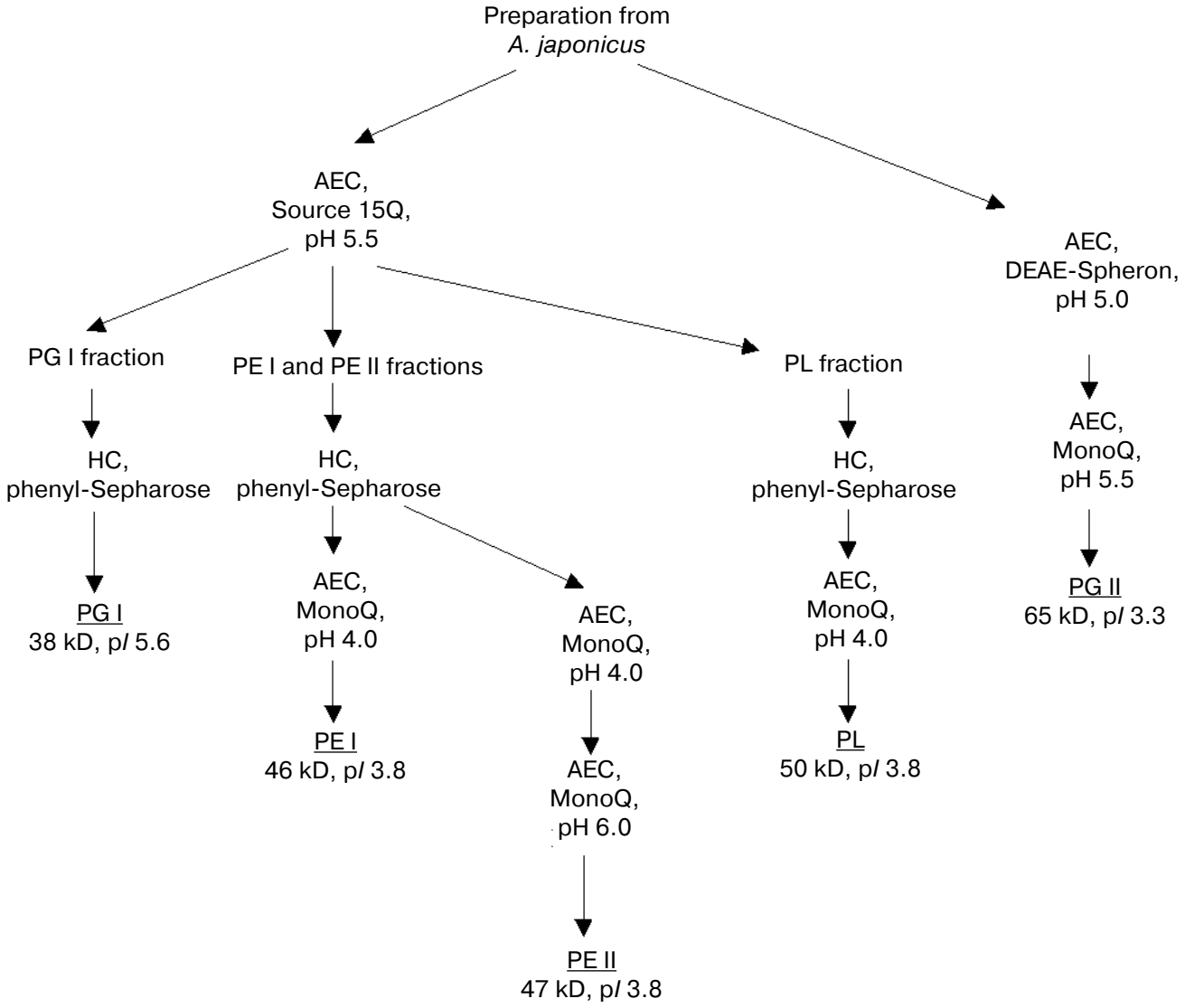

Fig. 1. Scheme of isolation of the enzymes. AEC, anion-exchange chromatography; HC, hydrophobic chromatography; PG, polygalacturonase; PL, pectin lyase; PE, pectinesterase.

40 cm, Chemapol, Czechia) was employed using an Econo-System low-pressure chromatographic system (BioRad, USA). The enzyme preparation was desalted on the P2 Acrylex column in 50 mM sodium-acetate buffer, pH 5.0. The sample containing 1.5 g of the protein was applied to the DEAE-Spheron column equilibrated with the same buffer. The column was washed with the starting buffer, and the bound protein was eluted with a 0-0.5 M linear NaCl gradient at 4 ml/min (gradient volume, 3 liters). The fraction eluted at 0.31 M NaCl containing polygalacturonase II was passed through the P2 Acrylex column equilibrated with 20 mM piperazine-HCl buffer, pH 5.5, and then subjected to anion-exchange chromatography on the Mono Q HR 5/5 column equilibrated with the same buffer. The Mono Q column was washed with the starting buffer, and the bound protein was eluted with 60 ml of a 0-0.4 M linear gradient of NaCl at 0.5 ml/min.

Determination of biochemical characteristics of the individual enzymes. Analytical isoelectric focusing of proteins was performed using a Model 111 Cell unit (BioRad) according to the user's manual. Electrophoresis was performed in 12% polyacrylamide gel on a Mini Protean unit (BioRad) according to the manual. Protein bands in the gels were stained with Coomassie Blue G-250 or with silver.

Changes in the molecular weight distribution of the products of the hydrolysis of polygalacturonic acid were

studied on a Protein Pak 125 gel-penetrating chromatography column (Waters, USA) using a Workstation 700 chromatography system (BioRad) for HPLC equipped with a refractometric detector. The eluting buffer was 50 mM acetate, pH 5.0, containing 0.1 M NaCl.

RESULTS

Isolation of pectinases. The scheme of the purification of pectinases of *A. japonicus* is presented in Fig. 1. Using Source 15Q anion-exchange chromatography and subsequent steps including phenyl-Sepharose hydrophobic chromatography and Mono Q anion-exchange chromatography, four enzymes of the pectinase complex were isolated: polygalacturonase I, pectinesterases I and II, and pectin lyase. In the case of the fifth component (polygalacturonase II), the first stage was DEAE-Spheron anion-exchange chromatography. It should be noted that originally DEAE-Spheron was employed in the first stage of purification for all the indicated enzymes, the procedure resulting in isolation of the partially purified preparations. However, later we changed this carrier to Source 15Q that resulted in faster and more effective purification. The disadvantage of Source 15Q was less capacity of the column and the impossibility of loading of large amounts of the preparation. Therefore, the primary purification was performed in three batches, and then the analogous fractions were pooled.

The use of Source 15Q chromatography in the first stage of purification yielded several fractions containing the polygalacturonase activity (Fig. 2). The main activity was revealed in the fraction of the unbound protein (fraction *1* in Fig. 2). This fraction was used to isolate homogenous polygalacturonase I using hydrophobic chromatography (the protein peak eluted at 1.1 M ammonium sulfate).

In the case of using DEAE-Spheron in the first stage, a significant level of the polygalacturonase activity was also observed in the fraction corresponding to 0.31 M NaCl after the bound protein was eluted with the salt gradient. This fraction was used to isolate polygalacturonase II using Mono Q anion-exchange chromatography. Besides, at least two minor fractions exhibiting polygalacturonase activity were also obtained during the purification, but they were neither investigated nor subjected to further purification.

Using Source 15Q chromatography, the following fractions were revealed: a fraction exhibiting pectinesterase activity that was eluted at 0.21 M NaCl (fraction *2* in Fig. 2) and a fraction with pectin lyase activity that was eluted at 0.3 M NaCl (fraction *3* in Fig. 2). These fractions were used to isolate the pectinesterases I and II and pectin lyase. No difficulties were found in the subsequent purification of the pectin lyase: the homogenous enzyme was obtained using sequential chromatographies of the corresponding fraction on the columns containing phenyl-Sepharose and Mono Q (Fig. 3).

The situation was more complicated in the case of the pectinesterase fraction. The original pectinesterase fraction (after Source 15Q) contained many protein bands as revealed by SDS-PAGE. Phenyl-Sepharose hydrophobic chromatography yielded two similar fractions with pectinesterase activity: the first fraction was eluted at 1.3 M ammonium sulfate, and the second at 1.1-0.9 M ammonium sulfate. Both fractions yielded several protein bands on SDS-PAGE. The first fraction possessed 2-fold higher specific activity. This stage appeared to be very efficient, allowing separation of significant

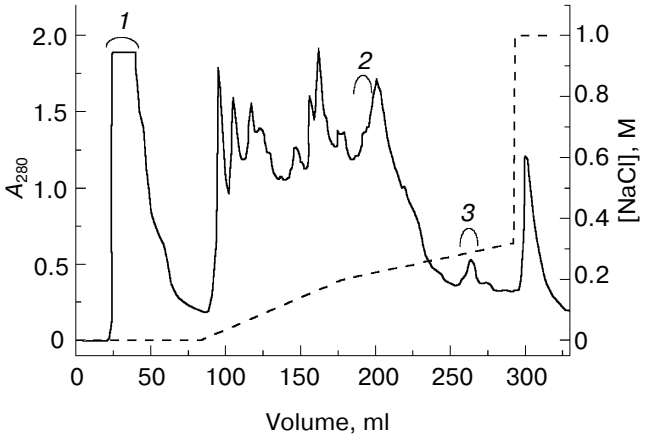

Fig. 2. Fractionation of the original enzyme preparation on a Source 15Q column: *1*) polygalacturonase fraction; *2*) pectinesterase fraction; *3*) pectin lyase fraction. The gradient of the salt is shown by the dashed line.

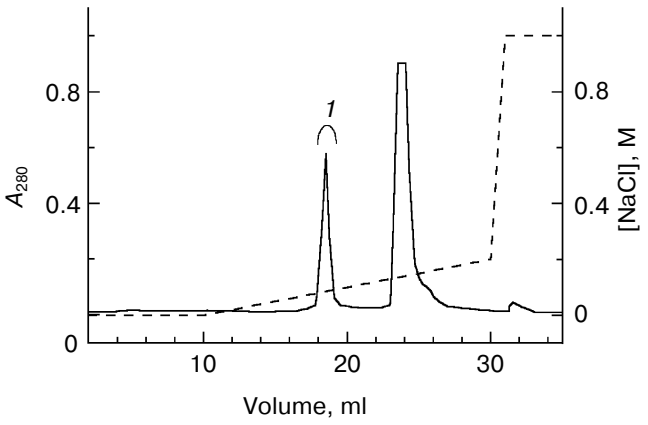

Fig. 3. Isolation of homogenous pectin lyase (*1*) using a Mono Q column. The salt gradient is shown by the dashed line.

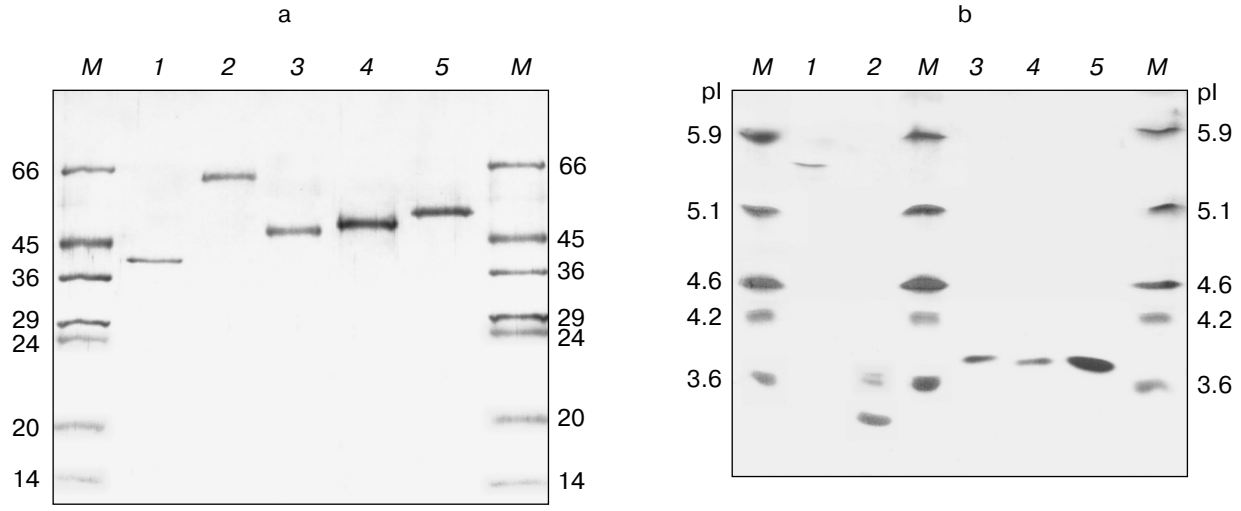

Fig. 4. Electrophoresis (a) and isoelectric focusing (b) of the isolated enzymes: polygalacturonase I (*1*), polygalacturonase II (*2*), pectinesterase I (*3*), pectinesterase II (*4*), and pectin lyase (*5*). Molecular weights of protein markers in kD (a) and their p*I* values (b) are shown on the left and on the right.

amount of extraneous proteins that resulted in 10-20-fold increase in the specific activity. Then these fractions were subjected to Mono Q anion-exchange chromatography at pH 4.0. Pectinesterase I was eluted at 0.073 M NaCl as a well separated peak and was homogeneous. Pectinesterase II was eluted at 0.085 M NaCl as a group of peaks. However, most of the extraneous proteins were removed. The resulting fraction gave two main protein bands (47 and 64 kD) as shown by electrophoresis. It was subjected further to Mono Q chromatography at pH 6.0 to yield the homogenous pectinesterase II.

Main properties of the purified pectinases. The purification of the enzyme preparation from *A. japonicus* yielded five enzymes involved in degradation of pectin substances: two polygalacturonases (I and II), pectin lyase, and two pectinesterases (I and II). The yields in the activities and the final degrees of purification are presented in Table 1. The molecular weights and isoelectric points of the enzymes were determined using SDS-PAGE and isoelectric focusing in polyacrylamide gel (Fig. 4 and Table 1). All the enzymes were homogenous and yielded single bands on the corresponding gel plates, except for polygalacturonase II that exhibited two bands on isoelectric focusing (but one band on the electrophoregram).

The substrate specificity of the isolated enzymes was determined using a number of polysaccharide substrates. None of these enzymes possesses cellulase, xylanase, mannanase, galactanase, arabinase, or amylase activity,

Table 1. Biochemical characteristics of the isolated enzymes

Enzyme	Yield of main activity, %	Extent of purification	Molecular weight, kD	p*I*	Activity, U/mg			
					PGA (RS)	pectin (A_{232})	pectin (viscometry)	pectin (pH-stat)
Polygalacturonase I	26	64	38	5.6	117	0	42	0
Polygalacturonase II	5	5	65	3.3	13	0	9.3	0
Pectinesterase I	4	732	46	3.8	0	0	0	805
Pectinesterase II	6	763	47	3.8	0	0	0	839
Pectin lyase	21	263	50	3.8	0	30	863	0

Note: PGA, polygalacturonic acid; RS, reducing sugars.

since no reducing sugars were revealed after incubation of the enzymes in the presence of carboxymethylcellulose, xylan, xyloglucan, galactomannan, galactan, linear and branched arabinans, and starch. Both polygalacturonases exhibited activity towards polygalacturonic acid, also reducing relatively slowly the viscosity of citrus pectin (Table 1). The specific activity of polygalacturonase I was much higher than that of polygalacturonase II. This was likely due to partial inactivation of the second enzyme during the purification. No other enzymatic activities were revealed in the preparations of the polygalacturonases. The pectin lyase exhibited high activity towards highly methylated pectin that was monitored by both the specific lyase reaction (accumulation of the unsaturated products at 232 nm) and the viscometric method (Table 1). However, this enzyme was inactive towards the completely demethylated substrate (polygalacturonic acid). The pectinesterases I and II cleaved the methoxyl bonds of highly methoxylated pectin, releasing the free carboxyl residues of the polygalacturonan chain that was detected both qualitatively by using a pH indicator and quantitatively using a pH-stat (Table 1). The isolated pectinesterases I and II are probably isoforms of the same enzyme. This is supported by their similar molecular weights (46 and 47 kD), the same p*I* values (3.8), similar specific activities (805 and 839 U/mg), and similar behavior on chromatography: in the case of hydrophobic chromatography as well as in the case of anion-exchange chromatography, both pectinesterases were eluted as adjacent peaks. The pH-dependences of the activities were virtually the same for the two enzymes (Fig. 5b). For further investigations of the functioning of the pectinase complex we pooled pectinesterases I and II.

The dependences of the enzymatic activities of the isolated enzymes on pH are shown in Fig. 5 (a and b). All the enzymes had their maximal activity in an acid medium. Polygalacturonases I and II exhibited narrow pH optima in the region of pH of 5.0 and 4.0, respectively. The pectinesterases had maximal activities at pH 4.5. The pectin lyase exhibited a high activity in the range of pH 4.5-5.5.

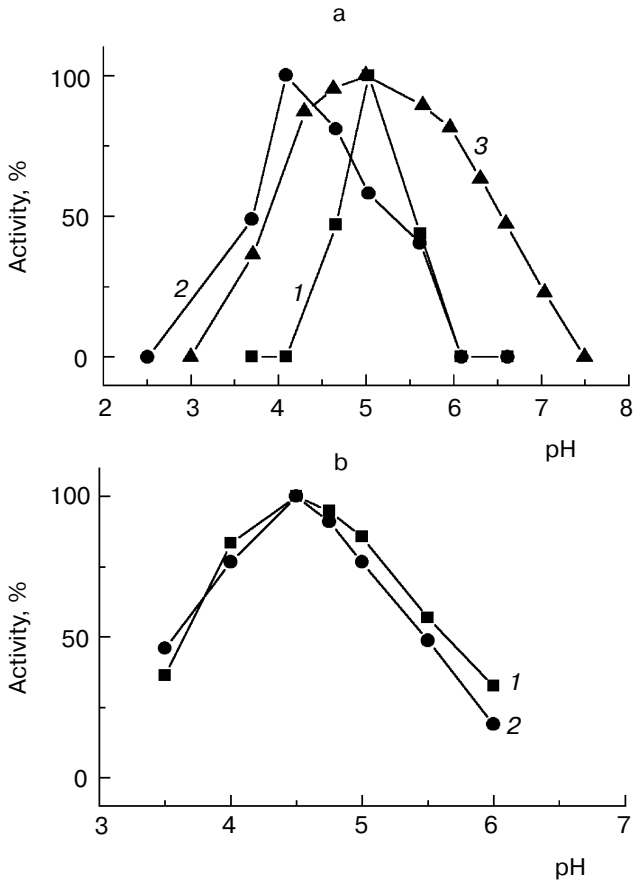

Fig. 5. pH dependences of the pectinase activities: a) polygalacturonases I (*1*) and II (*2*), pectin lyase (*3*); b) pectinesterases I (*1*) and II (*2*).

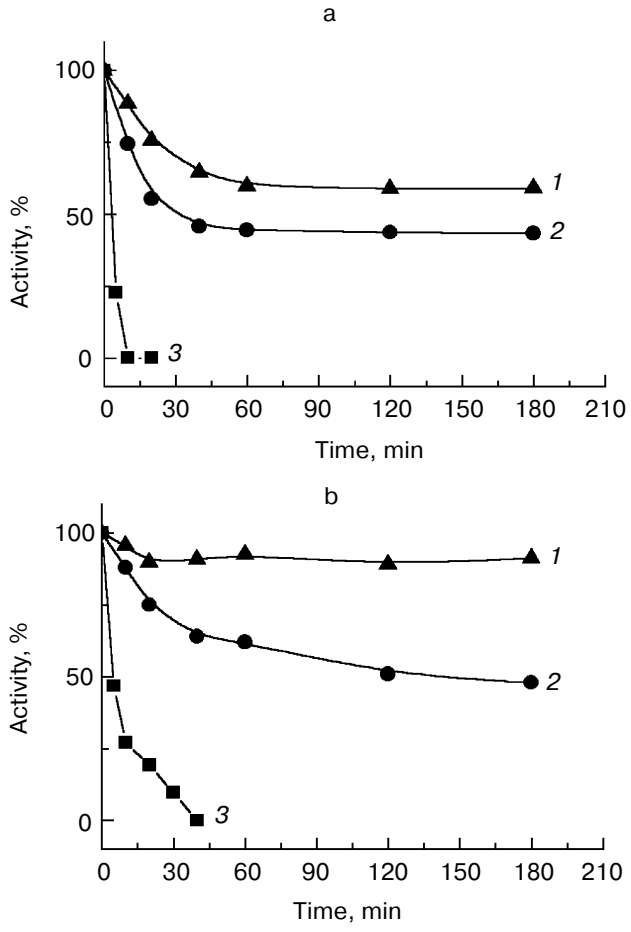

Fig. 6. Stability of polygalacturonases I (a) and II (b) at different temperatures: 30°C (*1*), 40°C (*2*), and 50°C (*3*).

Investigation of the thermal stability of the enzymes showed that both of the polygalacturonases are relatively unstable at higher temperature (Fig. 6). Both enzymes lost more than 50% of their activity during 5 min at 50°C and the optimal pH of the medium (5.0 and 4.0). At 30°C polygalacturonase II retained 90% of its activity after 3 h of incubation, and polygalacturonase I lost 40% of its activity after 1 h of incubation. The pectin lyase and the pectinesterase exhibited higher thermal stability than the polygalacturonases, retaining ~90% of their activity after 3 h of incubation at 50°C. After 3 h of incubation at 40°C, the enzymes completely retained their activity, and the incubation at 60°C for the same time resulted in 55 and >95% loss of the activity, respectively.

It is known from the literature [1, 13] that pectinases are often cation-dependent (particularly Na^+- and Ca^{2+}-dependent) enzymes. There is still no general theory concerning the effect of cations on the activity of pectinases. However, most researchers assume that mono- and bivalent cations affect mainly the substrate, changing its conformation, total charge, and extent of aggregation [1]. Figure 7 demonstrates the dependences of the activities of polygalacturonases I and II towards polygalacturonic acid on NaCl concentration. As seen from the data, the activity of both polygalacturonases strongly depends on concentration of NaCl, exhibiting maximal activity at 0.1-0.2 M. It was impossible to study the effect of Ca^{2+} on the polygalacturonase activity because the solution of the substrate was converted into a gel on the addition of even low concentrations of $CaCl_2$ (10 mM). Since chelating agents such as EDTA affect the concentration of free cations in solutions, the effect of EDTA on the reactions catalyzed by the polygalacturonases was studied. The activity of both polygalacturonases gradually decreased on increasing the concentration of EDTA from 1 to 10 mM. The activity was completely suppressed in the presence of 8-10 mM EDTA.

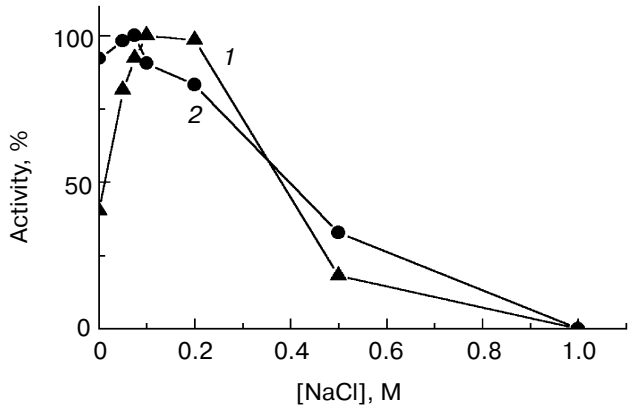

Fig. 7. Effect of NaCl on the activity of polygalacturonases I (*1*) and II (*2*).

In the reaction of the pectin lyase with highly methylated citrus pectin, no effect was observed in the presence of NaCl or $CaCl_2$ (at least while increasing the concentration of the salts from 0 to 1 and 0.1 M, respectively).

Kinetic behavior of the purified pectinases. The kinetic parameters (K_m and V_{max}) of the polygalacturonases in the reaction with polygalacturonic acid and those of the pectin lyase in the reaction with pectins with different content of the methoxyl groups were determined from the dependences of the initial rates of the reactions on substrate concentrations (at 40°C and the optimal pH values) using Lineweaver–Burk plots. The catalytic constants k_{cat} were calculated from the V_{max} values considering the protein concentrations in the reaction mixture and the molecular weights of the enzymes (Table 1). The values of the kinetic parameters are given in Table 2. Polygalacturonase II had a lower K_m value compared to that of polygalacturonase I, but the k_{cat} value was lower for polygalacturonase II as well. In the case of the pectin lyase, the K_m value decreased, and the k_{cat} value increased with increasing the methylation degree of pectin from 26 to 89%. Thus, this enzyme exhibited the highest specificity towards highly methylated pectin (MD, 89%), this confirming the correctness of the classification of the enzyme as a pectin lyase.

Table 2. Kinetic parameters of pectinases

Enzyme	Substrate	K_m, g/liter	k_{cat}, sec^{-1}
Polygalacturonase I	PGA	1.03	90
Polygalacturonase II	PGA	0.35	26
Pectin lyase	pectin, MD of 26%	2.4	20
Pectin lyase	pectin, MD of 65%	1.3	77
Pectin lyase	pectin, MD of 89%	0.9	86

Note: PGA, polygalacturonic acid; MD, degree of methylation of the carboxyl groups of pectin.

Kinetic curves of the hydrolysis of polygalacturonic acid in the presence of polygalacturonases I and II are shown in Fig. 8. The extent of the hydrolysis of the substrate 2 h after the beginning of the reaction was 15 and 20%, respectively. After 16 h of the reaction, the extent of the hydrolysis increased only slightly, to 18 and 25%, respectively. The addition of a fresh portion of the enzyme 16-17 h after the beginning of the reaction (shown by arrows) increased the yield of the reducing sugars for both polygalacturonases, indicating that the cessation of the hydrolysis of the substrate was due to inactivation of the

enzymes in spite of the relatively low temperature of the medium (30°C).

To determine the type of action of polygalacturonases I and II on polygalacturonic acid, the composition of the products during the hydrolysis of the substrate was analyzed using gel-penetrating chromatography on a Protein Pak 125 column. Aliquots were taken from the reaction mixture after 6, 11, 30, 60, 90, and 120 min after the beginning of the reaction. The changes in the gel chromatograms of the products of the hydrolysis of polygalacturonic acid in the presence of polygalacturonase I are shown in Fig. 9. During the reaction, the area of the peak that corresponded to the high-molecular-weight substrate decreased, while the products of the hydrolysis of a lower weight accumulated in the region that corresponded to oligomeric compounds. Similar behavior was observed in the case of polygalacturonase II. Such character of the molecular weight distribution of polysaccharides during the reaction is characteristic for enzymes of endo-depolymerizing type. Thus, we can conclude that both isolated polygalacturonases are endo-polygalacturonases in terms of their mechanism of action.

In the case of the pectin lyase, the kinetics of the cleavage of citrus pectins of different methylation degree was studied (Fig. 10). The pectin lyase acted most effectively on highly methylated pectin (MD 89%) and destroyed very slowly low methylated pectin (MD 26%). When the reaction was complete, the extent of the conversion for pectins of 26, 65, and 89% of methylation degree constituted 2, 10, and 16%, respectively. The addi-

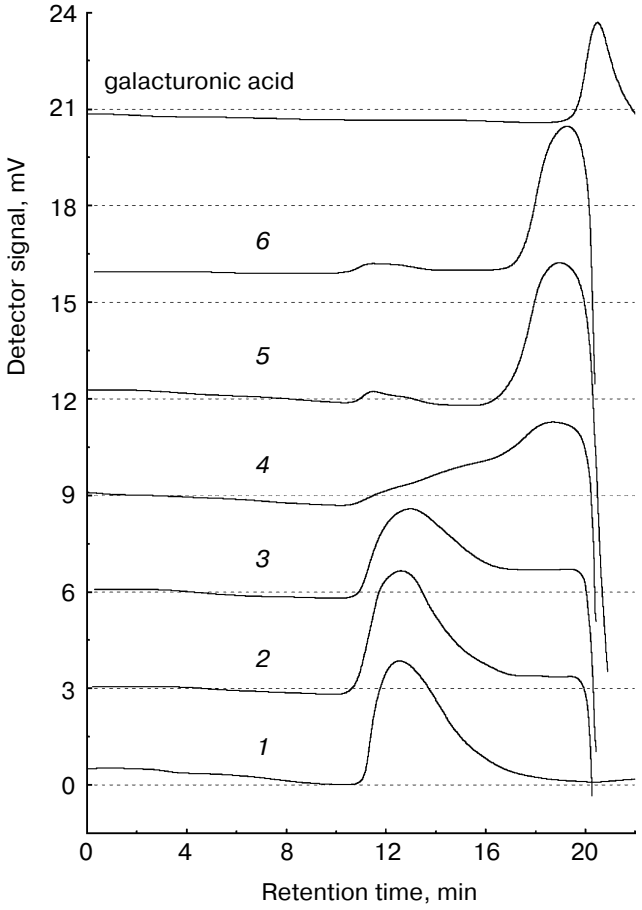

Fig. 9. Gel chromatograms of the original substrate and the products of the hydrolysis of polygalacturonic acid in the presence of polygalacturonase I at different reaction time (portion of the hydrolyzed bonds): 0 (*1*), 6 (1.7%) (*2*), 11 (2.5%) (*3*), 30 (6.7%) (*4*), 60 (11.9%) (*5*), and 120 min (14.5%) (*6*).

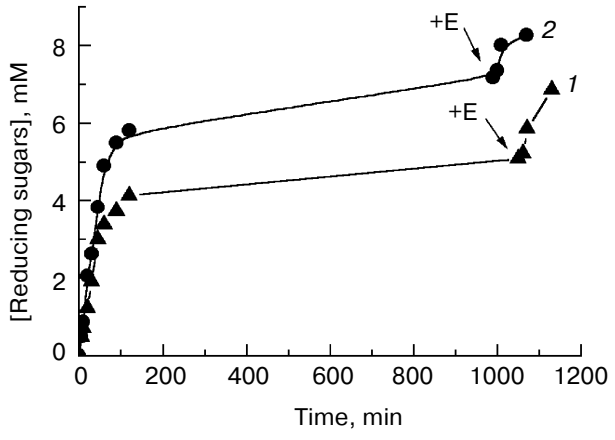

Fig. 8. Accumulation of the products of hydrolysis of 0.5% polygalacturonic acid in the presence of polygalacturonases I (*1*) and II (*2*) at 30°C and pH 5.0 (*1*) or 4.0 (*2*). Additions of fresh portions of the enzyme in the reaction mixture are shown by the arrows.

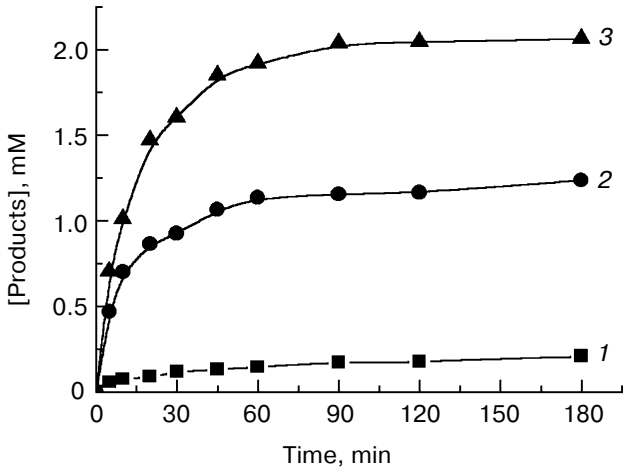

Fig. 10. Accumulation of Δ-4,5-unsaturated products of destruction of pectins (0.22%) in the presence of the pectin lyase (40°C, pH 4.5). The degree of methylation was 26 (*1*), 65 (*2*), and 89% (*3*).

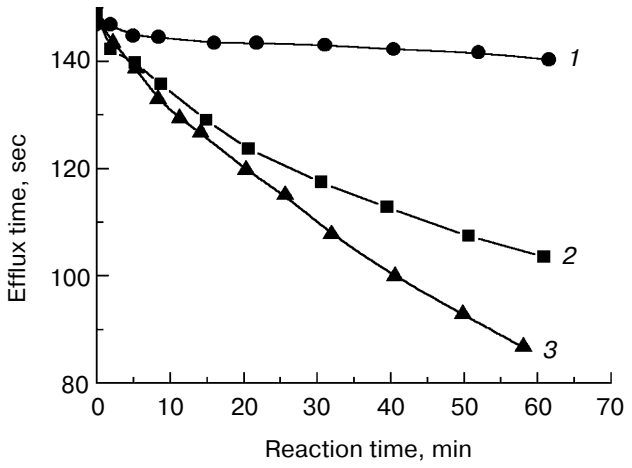

Fig. 11. Change in the viscosity of 0.5% pectin solution in the presence of pectinesterase (*1*), polygalacturonase II (*2*), and the mixture of these enzymes (*3*) at the ratio of 1 : 1 (protein content) at 32°C and pH 4.0.

tion of a fresh portion of the enzyme did not increase the concentration of the unsaturated products, indicating that the cessation of the reaction was not due to the enzyme inactivation. These results agree well with the kinetic parameters (K_m and k_{cat}) calculated from the initial rates of the reaction (see above), according to which the pectin lyase cleaves preferably highly methylated pectins. Obviously, the isolated pectin lyase was also an endo-depolymerase in terms of the reaction mechanism, since it decreased effectively the viscosity of pectin.

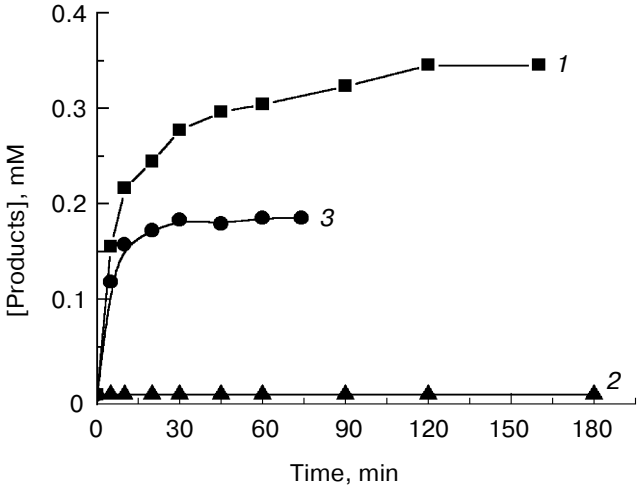

Fig. 12. Accumulation of Δ-4,5-unsaturated products of the destruction of pectin (0.06%, methylation degree ~70%) in the presence of pectin lyase (*1*), pectinesterase (*2*), and the mixture of these enzymes (*3*) at the ratio of 3.5 : 1 (protein content) at 40°C and pH 4.5.

Synergism and antagonism during joint action of the enzymes of the pectinase complex. It is known that polygalacturonases involved in degradation of the main chain of pectin are able to act in synergism with pectinesterase [11]. The cause of the synergism is the fact that the esteric groups of the pectin chain can hinder polygalacturonases in their acting. The pectinesterase activity results in demethylation of the carboxyl residues that accelerates the hydrolysis of the main chain of the pectin molecule by polygalacturonase. At the same time, it can be assumed that the demethylation of pectin by pectinesterase must decrease the rate and the extent of pectin degradation by pectin lyase.

To study the interaction of the polygalacturonases and the pectinesterase, the kinetics of the changes in the viscosity of a pectin solution was investigated in the presence of one of the polygalacturonases, pectin-esterase, or their mixture (1 : 1). Figure 11 shows the corresponding data for polygalacturonase II. As seen from the figure, the individual pectinesterase did not change the viscosity of the pectin solution. In the presence of individual polygalacturonase II, the viscosity of the solution decreased, and on the addition of the pectinesterase into the reaction mixture containing the polygalacturonase II the rate of the decrease in the viscosity of the pectin solution increased, this indicating a synergism between the pectinesterase and the polygalacturonase. Similar behavior was observed in the case of the joint action of polygalacturonase I and the pectinesterase (data not shown). For mixtures of polygalacturonase II and the pectinesterase taken in different proportions the coefficients of synergism were determined. The maximal value of the synergism coefficient was 2.1 at the ratio of the enzymes 3 : 1 in terms of protein concentrations.

To study the interaction between the pectin lyase and the pectinesterase, the kinetic curves of destruction of highly methylated pectin in the presence of the individual enzymes and their mixture at the ratio of 3.5 : 1 were analyzed (Fig. 12). The pectinesterase was incapable of cleaving the glycoside bonds of pectin yielding unsaturated products. The pectin lyase destroyed pectin effectively, and 2.5 h after the beginning of the reaction the extent of the substrate conversion reached 9%. As was expected, the presence of the pectinesterase in the reaction mixture decreased the rate of the reaction and lowered the extent of the substrate conversion to 6%. Thus, an antagonism was observed under the joint action of the pectinesterase and pectin lyase.

DISCUSSION

According to the literature, pectinases from different species of *Aspergillus* can differ significantly in their bio-

chemical parameters: the molecular weight of polygalacturonases varies from 36 to 83 kD, and the p*I* value varies from 3.2 to 8.3 [14-23]. There are seven different genes in the genome of *A. niger* encoding endo-polygalacturonases, and six of them have already been described [23]. In the present work two polygalacturonases were isolated from *A. japonicus*. The enzymes had molecular weights of 38 and 65 kD and p*I* values of 5.6 and 3.3.

The study of the change in the molecular weight distribution of polygalacturonic acid during the hydrolysis showed that both polygalacturonases were endo-depolymerases in terms of the mechanism of their action on the polymeric substrate. The enzymes exhibited maximal activity in the narrow range of pH and possessed relatively low thermal stability that is typical for many of the fungal polygalacturonases described in the literature. The characteristic feature of the isolated polygalacturonases was that their activity depended strongly on NaCl concentration. Despite the fact that both polygalacturonases were capable of decreasing the viscosity of highly methylated pectin (MD ~ 70%), the rate of the reaction was low. On the addition of the pectinesterase into the reaction mixture, the rate of the decrease in the substrate viscosity increased. Thus, the polygalacturonases isolated from *A. japonicus* acted on the non-esterified glycoside residues of polygalacturonan containing the free carboxyl groups, exhibiting the properties of typical endo-polygalacturonases.

The isolated isoforms of pectinesterase (46 and 47 kD, p*I* 3.8) had molecular parameters similar to those of the enzyme from related *A. aculeatus* [11] that has molecular weight of 43 kD (as shown by SDS-PAGE) and p*I* 3.8. Besides, both isolated pectinesterases exhibited maximal activity at the same pH (4.5). Interestingly, in the case of *A. aculeatus* a small amount of an isoform of this enzyme that differed slightly in molecular weight was also revealed [11]. Pectinesterases exhibiting similar biochemical parameters and properties were isolated earlier from *A. niger* [24, 25] and *A. oryzae* [26].

The pectin lyase isolated in the present work had molecular weight of 50 kD and p*I* value of 3.8. The enzyme exhibited maximal activity in the range of pH 4.5-5.5, affected preferably highly methylated pectins, hydrolyzed weakly low methylated pectin (MD 26%), and did not affect polygalacturonic acid. The enzyme differed significantly in its properties from the pectin lyases of *Aspergillus* described in the literature, which had lower molecular weight (32-38 kD), differed in values of p*I*, and exhibited different pH optima [13, 27-30]. For example, pectin lyase from *A. japonicus* described previously had p*I* value of 7.7 and exhibited maximal activity at pH 7.0 [27]. Pectin lyase B from *A. niger* had molecular weight of 38 kD and exhibited maximal activity at pH 8.5 [28]. Pectin lyase isolated from the commercial preparation Cytolase PCL5 (*A. niger*) was similar to the enzyme isolated in the present work in terms of p*I* value (3.6), but it had lower molecular weight (38 kD), and its activity was maximal in the range of pH 5.8-6.4 [29, 30].

While acting simultaneously on highly methylated pectin, the isolated pectin lyase and pectinesterase exhibited antagonism. This fact is understandable considering the data on the specificity of pectin lyase towards the substrates with different degree of methylation. Considering the fact that in the case of the enzyme pair polygalacturonase–pectinesterase synergism was observed, we can conclude that pectinesterase likely plays an important regulatory role in the metabolism of *Aspergillus*.

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