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Penicillium sp. 23 alpha-galactosidase: purification and substrate specificity

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Abstract

α-Galactosidase, a glycoprotein with carbohydrate and protein in ratio 1:6, has been isolated from liquid culture of micromycete *Penicillium* sp. 23 and purified to homogeneous state by ammonium sulphate precipitation followed by ion exchange and gel-filtration chromatography on TSK-gels. The *Penicillium* sp. 23 α-galactosidase specificity against a series of natural and synthetic substrates has been studied. The enzyme was found to exhibit strict specificity towards the glycon and hydrolyze exclusively α-D-galactosides such as *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPhGal), melibiose, raffinose and stachyose. The configuration at C1 and C4 atoms of substrate as well as substitution at C2 and C6 of substrate made an important contribution to the interaction with the enzyme. The tested α-galactosidase exerted the highest affinity (K_m) with respect to the synthetic substrate *p*-NPhGal and maximal rate of hydrolysis (V_{max}), about 10 times higher, comparing with natural substrates (melibiose, raffinose and stachiose). The *Penicillium* sp. 23 α-galactosidase possesses wide specificity towards α-galactosidase hydrolysis link type, splitting off at varying rates the terminal galactose from disaccharides, attached by α-1,2-, α-1,3- and α-1,6-links. The enzyme is ineffective towards disaccharides with α-1,4-link. The enzyme showed potential to splitting off α-1,3-bound terminal galactose residues from antigens of the human blood group B(III) erythrocytes. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Penicillium* sp. 23; α-Galactosidase; Substrate specificity

1. Introduction

At present the problem of creating a universal donor blood is extra-ordinarily important, especially in connection with the Chernobyl accident, which has led to increase in blood diseases. One of the ways to solve this problem is usage of enzymes, which strip blood erythrocytes of their group specificity. In this connection attention was paid to α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.2), which hydrolyzes the terminal nonreducing α-D-galactose residues in oligo-, or polysaccharides, glycolipids and glycoproteins.

Most of the known α-galactosidases hydrolyze only α-1,6-galactosides (Ohtacara, Mitsutomi & Uchida, 1984; Zaprometova, Ulezlo, Lichosherstov & Martinova, 1990), just as some of them (for instance from *Aspergillus awamori*) (Neustroev, Krilov, Abroskina, Firsov, Nasonov & Chorlin, 1991) are also capable of removing α-D-galactose residues from the trisaccharide: Galα-1,3-(Fucα-1,2)-Gal, which determines group B(III) specificity of human erythrocytes. α-Galactosidase from green coffee beans (Goldstein, Siviglia, Hurst & Lenny, 1982) and taro (Chien & Lin-Chu, 1991) splits the terminal α-1,3-linked galactose from membrane glycoproteins of blood group B(III) erythrocytes to strip their group-specific activity.

However, in spite of the numerous investigations, preparations of microbial α-galactosidases, which may be used in medicine to obtain universal donor blood, are unknown till now.

As the result of earlier screening of viable culture collection of the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine (Buglova et al., 1990), we selected micromycete *Penicillium* sp. 23 for producing α-galactosidase in liquid culture.

Therefore, the purpose of the present study was the isolation, purification of *Penicillium* sp. 23 α-galactosidase and investigation of its substrate specificity.

2. Materials and methods

2.1. Chemicals and reagents

All inorganic chemicals and organic solvents were reagent grade or better. BSA was obtained from “Sigma”;

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DEAE-Toyopearl 650 M and Toyopearl HW-55 TSK-gels were obtained from "Toyo Soda", Japan. The following substrates were used for investigations: *p*-nitrophenyl-α-D-galactopyranoside (*p*-NPhGal) (Sigma), *p*-NPh-β-D-galactopyranoside (Sigma), *p*-NPh-α- and β-D-glucopyranoside (Sigma), *p*-NPh-α-D-fuco-pyranoside ("Koch-Light"), *p*-NPh-*N*-acetyl-α-D-galactosaminide (Sigma), pNPh-*N*-Ac-β-D-glucosaminide (Koch-Light), *p*-NPh-*N*-Ac-β-D-galactosaminide (Sigma), melibiose (Sigma), raffinose (Sigma), stachyose (Sigma), disaccharides Galα-1,2-Gal, Galα-1,4-Gal (Sigma), Galα-1,3-Gal (received from the Department of Carbohydrate Chemistry, Institute of Organic Chemistry of the Russian Academy of Sciences).

2.2. Culture medium and culture conditions

Penicillium sp.23 was received from the culture collection of the Institute of Microbiology and Virology, National Academy of Sciences of Ukraine. The organism was grown on optimized medium (Buglova, Malanchuk, Ellanskaya, Zakharova & Cybien, 1993) at 28°C on a rotary shaker.

2.3. Enzyme purification

Mycelium was separated from the culture liquid by centrifugation. The supernatant was precipitated with ammonium sulfate at 30% of saturation and incubated for 2 h at 4°C. The pellet was concentrated by centrifugation for 30 min and discarded. To the supernatant was added dry ammonium sulfate to 90% of saturation, pH adjusted to 4.5–5.0 with 25% of NH_3 solution and kept for 10–12 h at 4°C. The precipitate was recovered via centrifugation, dissolved in water and dialyzed against 0.01 M phosphate buffer, pH 7.0.

Further α-galactosidase preparation purification was carried out both on the neutral and charged TSK-gels. Dialyzed α-galactosidase preparation from *Penicillium* sp. 23 was applied to a column (3×36 cm^2) with DEAE-Toyopearl 650 M equilibrated with 0.01 M phosphate buffer, pH 7.0 and washed by the same buffer. The absorbed proteins were eluted with the starting buffer in NaCl linear gradient from 0 to 0.8 M (flow rate being 24 ml/h). Fractions showing the α-galactosidase activity were collected, pooled and dialysed against the starting buffer. Evaporated material was applied to column (1.7×30 cm^2) with Toyopearl HW-55, equilibrated with 0.01 M phosphate buffer, pH 7.0 and the proteins eluted with the same buffer, containing 0.01 M NaCl (flow rate being 60 ml/h). Rechromatography was performed on an analytic column (1×5.6 cm^2) with DEAE-Toyopearl 650 M, equilibrated with 0.01 M phosphate buffer pH 6.8, elution was run with the same buffer in NaCl linear gradient between 0 and 0.8 M at 9 ml/h rate.

2.4. Electrophoretic procedure

Disc-electrophoresis in polyacrylamide-gel (PAGE) was carried out at pH 8.9 by the Davis procedure (Davis, 1964), using facilities and reagents from "Reanal" (Hungary). Electrophoresis was performed at 4 mA of current power per tube for 4 h; 50 μg of protein was plotted in one tube. The protein components were stained with 2.5% Coomassie R-250, the carbohydrate ones, with the Shiff reagent. The control gels were cut and extracted with 0.1 M phosphate–citrate buffer at pH 4.6 followed by the activity estimation.

2.5. Protein determinations

Protein was assayed by absorbance measurements at 280 nm and by the method of Lowry et al. (Lowry, Rosebrought, Farr & Randall, 1951) with bovine serum albumin as standard.

2.6. Carbohydrate determination

Carbohydrate was determinated by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1956).

2.7. Enzyme activity assays

When nitrophenylglycosides were used as substrates, the reaction mixture contained 0.1 ml of enzyme solution, 0.2 ml of 0.1 M phosphate–citrate buffer (PCB), pH 4.7, and 0.1 ml of 10 mM appropriate nitrophenylglycoside solution in the same buffer. The mixture was incubated for 10 min at 37°C, and then the reaction was stopped by the addition of 2 ml of 1 M Na_2CO_3 solution. The amount of *p*-nitrophenol released after enzymatic cleavage was estimated by the absorbance at 405 nm and calculated according to the standard curve. When the purified α-galactosidase preparation was used for hydrolysis of all nitrophenylglycosides, the concentration of enzyme preparation was 1 μg in pattern.

The unit (U) of α-galactosidase and other glycosidases activities was defined as the amount of enzyme which hydrolyzes 1 μmol of the substrate per minute under the conditions of the experiment.

2.8. Enzyme effect on the di-, tri- and tetrasaccharides

When melibiose and raffinose were used as substrates the reaction mixture contained 0.1 M PCB, pH 4.7, 5 mM of appropriate substrate and 5 μg (1.45 U) of α-galactosidase in a total volume of 1.5 ml. The samples were incubated for 2 h at 37°C and reaction was terminated by boiling. The amount of glucose (equivalent to the amount of galactose) liberated from melibiose was estimated by the glucose oxidase method (Bilai, 1973). The release of galactose from raffinose was measured by the method of Somogyi (1952).

The hydrolysis of disaccharides was carried out in the reaction mixture containing 40 nM of appropriate disaccharide in 0.1 M PCB pH 5.5, 0.02% sodium azide, solution of the α-galactosidase (0.05 U) in the same buffer, with the final volume being 0.4 ml. The mixture was incubated for 2 h at 37°C. The degree of disaccharide hydrolysis was

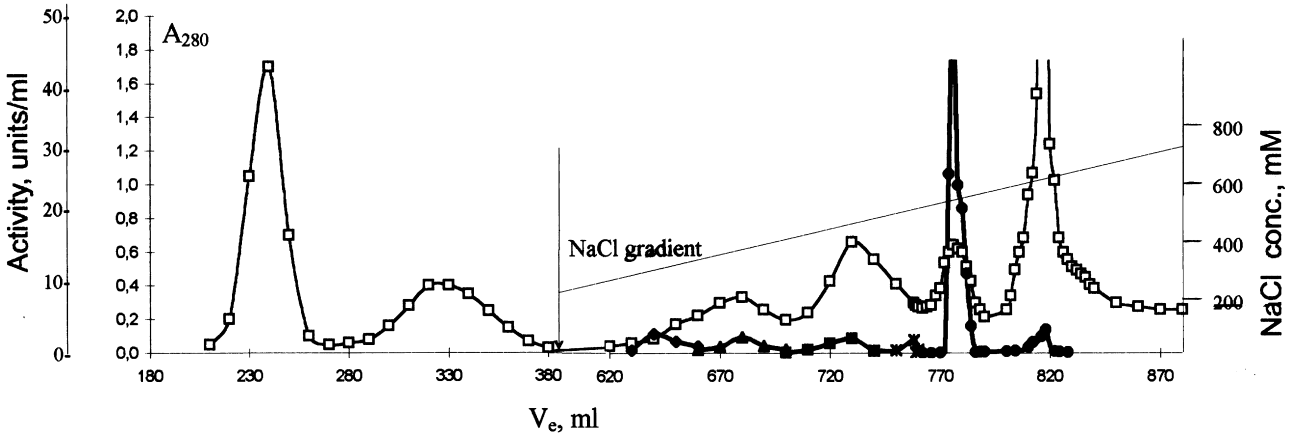

Fig. 1. Gradient elution column chromatography of *Penicillium* sp. α-galactosidase on DEAE-Toyopearl 650 M. Symbols: □, E_{280}; ●, α-galactosidase activity (fractions I and II); ◆, β-galactosidase activity; ▲, β-glucosidase activity; ■, α-*N*-Ac-galactosaminidase activity; H , β-*N*-Ac-glucosaminidase activity.

estimated by the amount of galactose liberated, which was identified using carbohydrate analyzer Biotronic LC-2000 (Germany) on the column (0.38×12.5 cm^2) with DA X8 (11 μm) in 0.5 M borate buffer, pH 8.0; the buffer flow rate was 27 ml/h. The amount of reducing sugars was determined with potassium bicinchoninate (Veremeychenko & Kishenko, 1991). The splitting of disaccharide during the incubation was also estimated by the decrease of its concentration in the reaction mixture.

2.9. Enzyme effect on the erythrocytes

While human blood group B(III) erythrocytes were used as substrates the reaction mixture contained 50 μl of suspension of group B(III) human erythrocytes fixed by glutaraldehyde, and solution of the enzyme (0.5 U) in 0.1 M PCB pH 5.5, 6.0, 6.5 and 7.3, the final volume was 175 μl. After 24 h of incubation the enzyme was removed from the reaction mixture and group B(III) erythrocyte-degrading activity was estimated using the hemagglutination inhibition test as described previously (Kuhlmann, Kovner & Rozenberg, 1986). The ability of α-galactosidase to remove group-specific determinants of group B (III) erythrocytes was evaluated according to the titer shift, at which hemagglutination occurs.

2.10. Relative enzyme efficiency estimation

In order to estimate how much effective α-galactosidase was from *Penicillium* sp. 23 relative to disaccharides and erythrocytes, an identical number of units of the enzyme under study and the commercial α-galactosdidase from the green coffee beans (“Sigma”) at 26°C and pH 5.5, 6.0, 6.5 and 7.3 were used.

2.11. Determination of kinetic parameters

In all cases the K_m and V_{max} values were determined by Lineweaver–Burk standard method (Berezin & Klesov, 1977).

3. Results and discussion

Since the culture liquid with the α-galactosidase activity showed insignificant β-galactosidase, β-glucosidase, α-*N*-acetyl-galactosaminidase and β-*N*-acetyl-glucosaminidase, the purification of α-galactosidase was performed. Its includes the following stages:

- step-by-step ammonium sulfate fractionation (30 and 90% of saturation) that resulted in partial purification from other glycosidases;
- ion-exchange chromatography on DEAE-Toyopearl 650 M (Fig. 1) that contributed to α-galactosidase preparation complete purification from accompanying glycosidases. Under conditions of NaCl gradient α-galactosidase appeared to be divided into two fractions (I and II);
- gel-filtration of α-galactosidase fractions I and II on Toyopearl HW-55 (Fig. 2) followed by further rechromatography of the pooled fractions on DEAE-Toyopearl 650 M (Fig. 3) resulted in the single protein peak showing α-galactosidase-specific activity of 290 U/mg of protein (protein content 0.2 mg/ml). Its homogeneity was supported by PAGE-electrophoresis (Fig. 3B). This fraction included carbohydrates as well as proteins in ratio 1:6. The glycoprotein nature of α-galactosidase preparation tested was confirmed by PAGE-electrophoresis (Fig. 3B).

The next step of our investigations involved the studies of α-galactosidase specificity.

It is known (Adya & Elbein, 1977; Kaneko, Kusakabe, Sakai & Murakami, 1990; Zaprometova et al., 1990) that α-galactosidases of diverse origin alongside with the natural substrates such as melibiose, raffinose and stachyose are

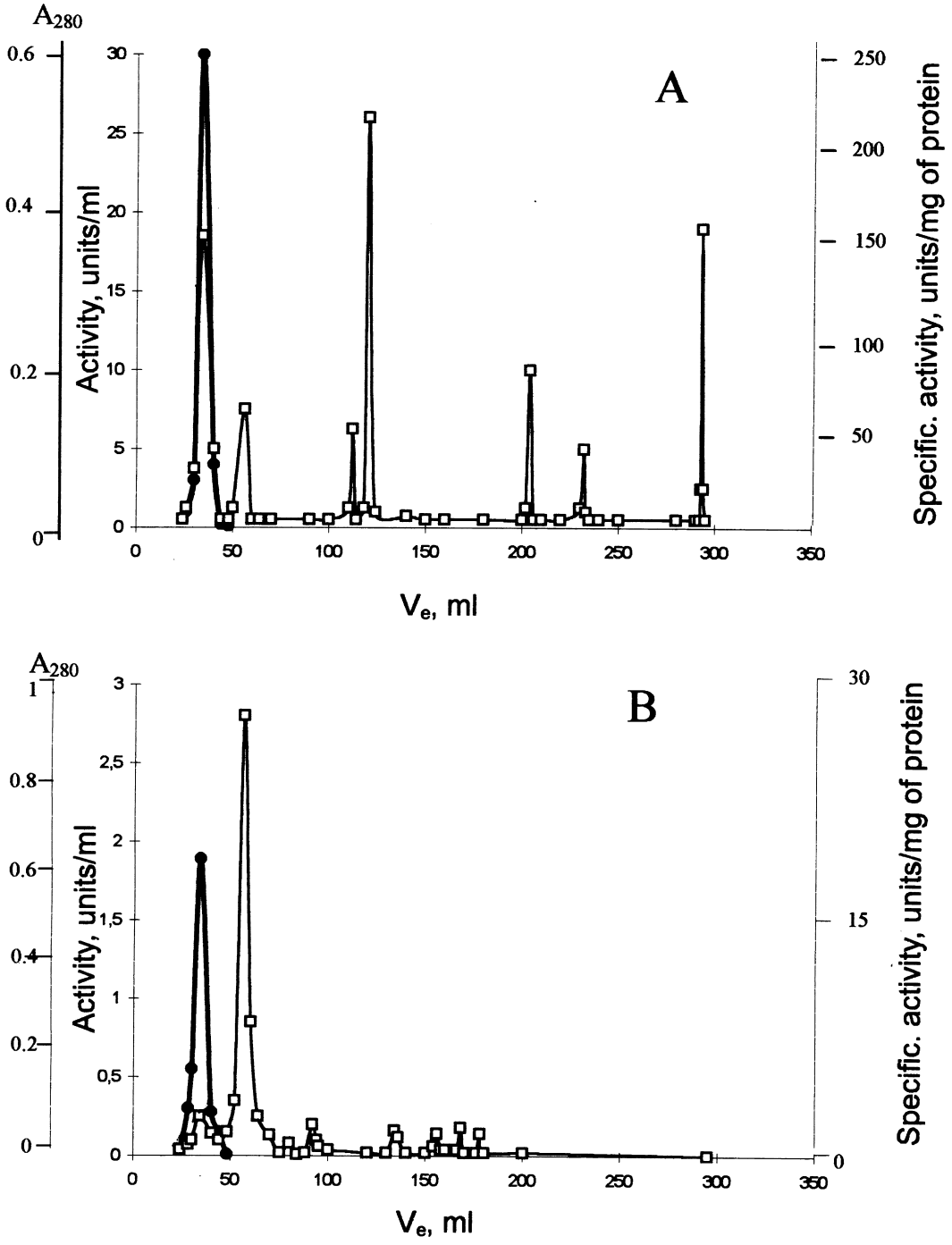

Fig. 2. Gel-filtration of *Penicillium* sp. α-galactosidase on Toyopearl HW-55: (A) fraction I of α-galactosidase; and (B) fraction II of α-galactosidase. Symbols: □, E_{280}; ●, α-galactosidase activity.

recognized to hydrolyze the synthetic *p*-nitrophenyl substrates as well, the latter being hydrolyzed much more extensively. Moreover, the α-galactosidase from *Aspergillus niger* is reported (Kaneko, Kusakabe, Ida & Murakami, 1991) to hydrolyze exclusively the synthetic substrates and failed to split off the terminal α-1,6-bound galactose in the linear structures like melibiose, raffinose, stachyose and galactomannans.

For specificity estimation of the α-galactosidase preparation from the *Penicillium* sp. 23, synthetic substrates were used (Table 1) carrying *p*-nitrophenol as an aglycon and α-D-galactose as a glycon (*p*-NPh-α-D-galactopyranoside), its stereoisomers at C1 and C4 (*p*-NPh-β-D-galactopyranoside, *p*-NPh-α-D-glucopyranoside) and derivatives with substituents at C2 (*p*-NPh-*N*-Ac-α-D-galactosaminide) and at C6 (*p*-NPh-α-D-fucopyranoside), as well as the natural substrates-

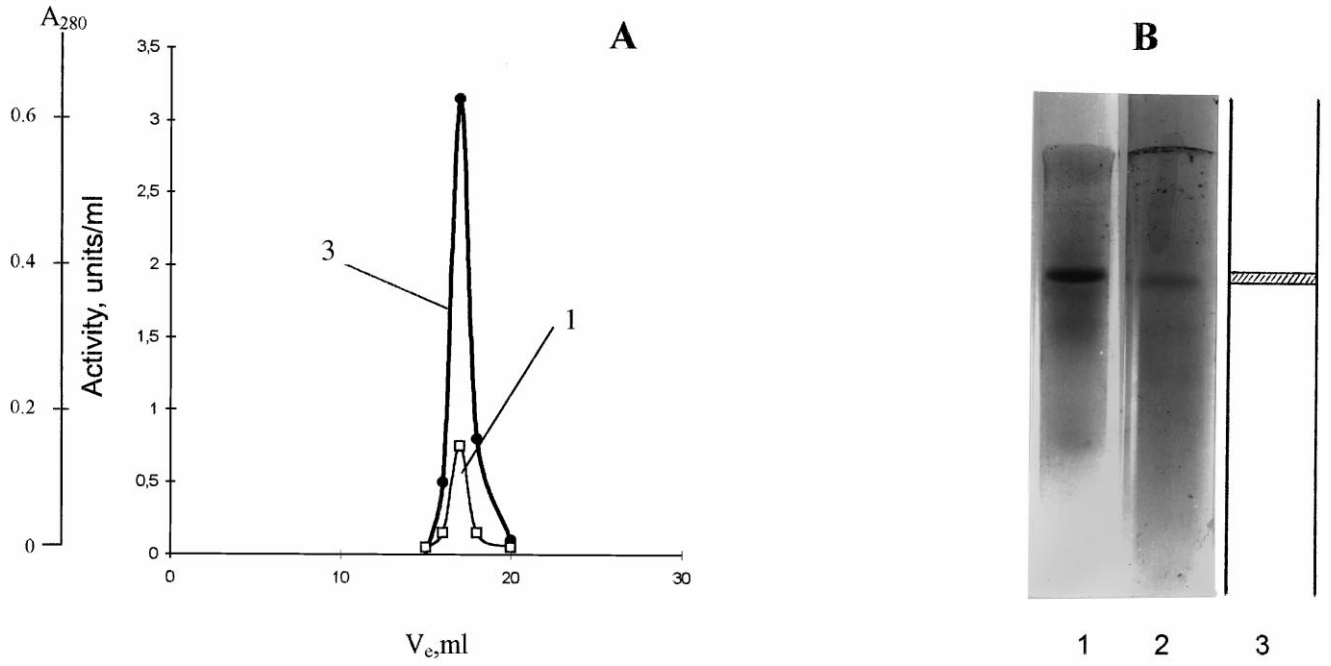

Fig. 3. (A) Rechromatography of *Penicillium* sp. α-galactosidase (fractions I + II) on DEAE-Toyopearl 650 M; and (B) PAGE patterns of native purified enzyme (50 μg) in 7.5% polyacrylamide gels. Symbol: 1, protein; 2, carbohydrate; 3, α-galactosidase activity.

melibiose, raffinose and stachyose, in which the terminal D-galactose is attached by α-1,6-link.

As is seen from Table 1, α-galactosidase from *Penicillium* sp. 23 can catalyze the hydrolysis exclusively of the α-D-galactosides and fails to hydrolyze *p*-nitrophenyl derivatives of β-D-galactose, α- and β-D-glucose, thus indicating the fact that the configuration at C1 and C4 atoms of the substrates is of utmost importance for their interaction with the enzyme. Similarly essential for mediation of the enzymatic reaction may be the substitution at C6 of substrate, since if the primary alcohol group is substituted for the methyl one the hydrolysis fails to occur (the enzymatic reaction with *p*-NPh-α-D-fucoside is lacking). α-Galactosidase under study is incapable as well to hydrolyze the substrates, bearing the substitutes at C2 position (*p*-NPh-*N*-Ac-β-D-glucosaminide, *p*-NPh-*N*-Ac-α- and β-D-galactosaminide).

It is worth noting that α-galactosidase from the *Penicillium* sp. 23 exhibits most affinity (K_m) to the synthetic substrate *p*-NPh-α-D-galactoside and its maximal rate of hydrolysis (V_{max}) is one order of magnitude higher than the hydrolysis rate of the natural substrates-melibiose, raffinose and stachyose (Table 1).

The literature data suggest that (Lazo, Ochoa & Gascon, 1977; Ohtacara et al., 1984) the K_m absolute value for the same substrate essentially varies with the enzyme origin. For example, K_m value for raffinose in α-galactosidase from *Aspergillus niger* constituted 0.5 mM, from *Pycnoporus*

Table 1
Substrate specificity of α-galactosidase from *Penicillium* sp. 23

Substrate	Structure	α-Galactosidase	
		V_{max} (U/mg of protein)	K_m (mM)
p-Nitrophenyl-α-D-galactopyranoside	Galα1-*p*-NPh	333.0	1.0
p-Nitrophenyl-β-D-galactopyranoside	Galβ1-*p*-NPh	0	-
p-Nitrophenyl-α-D-glucopyranoside	Glcα1-*p*-NPh	0	-
p-Nitrophenyl-β-D-glucopyranoside	Glcβ1-*p*-NPh	0	–
p-Nitrophenyl-*N*-Ac-α-D-galactosaminide	GalNAcα1-*p*-NPh	0	–
p-Nitrophenyl-*N*-Ac-β-D-galactosaminide	GalNAcβ1-*p*-NPh	0	–
p-Nitrophenyl-*N*-Ac-β-D-glucosaminide	GlcNAcβ1-*p*-NPh	0	–
p-Nitrophenyl-α-D-fucopyranoside	Fucα1-*p*-NPh	0	–
Melibiose	Galα1,6Glc	10.0	4.0
Raffinose	Galα1,6Glcβ1,2Fru	7.2	5.7
Stachyose	Galα1,6Galα1, 6Glcβ1,2Fru	9.1	3.5

Table 2
Specificity of α-galactosidases to the type of link to be split

Substrate	Amount of substrate (%) hydrolyzed by α-galactosidases	
	Penicillium sp. 23	Green coffee beans ("Sigma")
Galα1, 2Gal	47	28
Galα1, 3Gal	50	96
Galα1, 4Gal	0	9
Galα1, 6Gal	24	15

cinnabarinus 2.16 mM, from *Streptomyces* 9.6 mM, from *Saccharomyces carlsbergensis* 135.0 mM (Adya & Elbein, 1977; Lazo et al., 1977; Ohtacara et al., 1984; Oishi & Aida, 1976). A similar phenomenon is observed if melibiose is used as a substrate. However, most α-galactosidase preparations obtained by means of microbiological synthesis have in common the fact that they can hydrolyze the synthetic substrates (*p*-NPh-Gal) more extensively than the natural α-galactosides.

Investigations into the specificity of α-galactosidase from *Penicillium* sp. 23 as regards the pattern of the hydrolyzed link revealed (Table 2) that it can cleave the disaccharides in which the galactose is bound with α-1,2-, α-1,3- and α-1,6-links and is ineffective towards α-1,4-galactoside, while the α-galactosidase from coffee bean is able to split all the disaccharides, though the disaccharide with the α-1,4-link is hydrolyzed by the enzyme less effectively. The disaccharide with α-1,3-link is split by the α-galactosidase tested less effectively than coffee bean α-galactosidase, which is known (Goldstein et al., 1982) to be capable of inactivating the erythrocytes B(III) group specificity.

Similarly α-galactosidase from *Cephalosporium acremonium* exhibited a broad specificity towards the pattern of hydrolyzed link as well (Zaprometova et al., 1990), in contrast to its three isoforms isolated from the cells of protozoa *Trichomonas foetus* (Yates, Morgan & Watkins, 1975), each of which could hydrolyze a strictly definite type of α-D-galactoside link, namely, α-1,3-, α-1,4 or α-1,6- and be distinguished by the thermostability.

Presence of the side chains in the oligo- and polysaccharides could essentially affect the capacity of some α-galactosidases to hydrolyze the carbohydrate substrates. Thus, it was shown (Zaprometova et al., 1990) that α-galactosidase from *C. acremonium* could hydrolyze α-1,3-bound galactose in the disaccharides and could not split it off the branched trisaccharide Galα 1,3-(Fucα1,2)-Gal, found to be the group-specific determinant for the erythrocytes of the blood group B(III), while α-galactosidase from the green coffee beans could hydrolyze the linear and branched oligosaccharides carrying the terminal α-1,3-bound galactose residues.

Table 3
Titer of serum after stripping group B erythrocytes with α-galactosidases

Preparation	Titer of the stripped serum[a]			
	pH 5.5	pH 6.0	pH 6.5	pH 7.3
Penicillium sp. 23 α-galactosidase	1:32	1:32	1:16	1:16
Green coffee bean α-galactosidase	1:64	1:64	–	–

[a] Titer of original serum was 1:64.

Further studies on the specificity of *Penicillium* sp. 23 α-galactosidase were performed using the human B(III) erythrocytes fixed with glutaraldehyde as the natural high molecular weight substrate. It is known (Kuhlmann et al., 1986) that such fixed erythrocytes completely retain the group specificity, controlled by the terminal α-1,3-galactoside residues of the cell envelope glycoproteins.

Penicillium sp. 23 α-galactosidase proved to be susceptible to the structure of the terminal part of the group antigen B(III) similar to α-galactosidase from the green coffee beans. α-Galactosidase tested can inactivate B(III) group antigens, as is evidenced from the insignificant exhaustion of the anti-B-serum (titer shift only by 1–2 units) by B(III) erythrocytes, previously treated with α-galactosidase (Table 3). In the case of *Penicillium* sp. 23 α-galactosidase, the inactivating effect is more pronounced in the acidic pH range, which is consistent with its pH-optimum effect (Buglova & Malanchuk, 1995).

Besides, it should be mentioned that in α-galactosidase preparation tested fucosidase activity is lacking (Table 1), precluding the possibility of H-antigen inactivation through the enzymatic hydrolysis of their group-specific determinants during bioconversion of the type B(III) erythrocytes into O(I) type erythrocytes.

Thus, from the culture liquid of *Penicillium* sp. 23 was isolated, purified and characterized α-galactosidase with specific activity of 290 U/mg of protein. This activity significantly exceeds that of some commercial preparations of α-galactosidases. Our results demonstrated that *Penicillium* sp. 23 α-galactosidase can hydrolyze both natural and synthetic substrates, while exhibiting strict specificity towards the glycon and hydrolyzing, exclusively, α-D-galactosides. However, the enzyme possesses wide specificity towards α-galactosidase hydrolysis link type, splitting off with varying rates the terminal galactose, attached by α-1,2-, α-1,3- and α-1,6-links. In addition, the *Penicillium* sp. 23 α-galactosidase is able to split off the determinant α-1,3-bound galactose from B(III) group-specific antigens on human erythrocytes. This α-galactosidase attribute may become the prerequisite for its employment in hematological practice in an attempt at creating the universal donor blood.

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