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Amylase Inhibitors from *Streptomyces lucensis* VKPM Ac-1743 and *Streptomyces violaceus* VKPM Ac-1734

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Abstract—Inhibitors synthesized by the *Streptomyces lucensis* VKPM AS-1743 and *Streptomyces violaceus* VKPM AS-1734 strains were studied for their influence on amylases of different origin. The effect of the inhibitors was shown to be different on fungal amylase, pancreatic amylase, and amylase from human blood. It has been found that the studied inhibitors are substances of a pseudooligosaccharide nature and exhibit their activity and stability over a wide range of pH and temperature values. The physico-chemical and biochemical properties of isolated inhibitors were compared with those of known microbial inhibitors of α-glucosidases.

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INTRODUCTION

Simple sugars are formed as a result of the destruction of polysaccharides and are involved in complicated biochemical transformations in the cells of various organisms. An excessive level of these sugars leads to disruption of the carbohydrate balance. This can lead to diabetes and related complications, such as hyperglycemia and hyperlipidemia. To avoid this, sugar substitutes and sweeteners have been introduced into the diet, and physical activity has been combined with a diet that requires a careful approach to the particular individual [1–3]. An alternative way to solve these problens is glycomodulation, i.e., supplementing the diet with food additives or drugs based on glucosidases inhibitors. These compounds inhibit or repress the activity of digestive system enzymes, which are directly involved in hydrolytic cleavage of carbohydrates. Inhibitor action slows the absorption of sugars and decreases the content of glucose in blood. Microbial metabolites are of greatest interest. Compared to inhibitors of animal and vegetable origin, they have a broader specificity and activity spectrum [4, 5]. Isolated inhibitors of microbial originmay be divided, for convenience, into compounds of peptide and pseudosaccharide natures. The latter group is of special interest, because they are characterized by a variety of structural forms, a broad spectrum of activity, and stability with respect to changes in the pH and temperature [2, 6]. Acarbose synthesized by *Actinoplanes species* is a pseudosaccharide, the most thoroughly investigated and used in practice [6]. It reduces the adsorption of starch, dextrines, maltose, and saccharose. Based on acrabose, the glucobay preparation is developed by the Bayer corporation (Germany). Miglitol, emiglitat, and voglibose are recomended for prophylaxis of various violations of carbohydrate metabolism, regulation of the sugar level in blood, and dietotherapy [7–9]. These preparations inhibit the activity of α-glucosidases, which catalyze hydrolysis of oligo- and disaccharides. α-Amylase is the main enzyme at the first stage of polysaccharide cleavage. Compounds of a protein nature are generally known among efficient inhibitors of amylases [10].

Screening of strains of the *Streptomyces* genus from the collection in All-Russia Research Institute of Food Flavorings, Acids and Dyes (ARIFFAD), and their selection led to the isolation of two producers of amylases inhibitors.

The goal of the work was to study the properties of inhibitors synthesized by new strains of streptomycetes.

METHODS

The objects of the study were amylase inhibitors synthesized by *Streptomyces lucensis* VKPM Ac-1743 (Sharova N.Yu., Khodkevich O.A., Pozdnyakova T.A.) and *Streptomyces violaceus* VKPM Ac-1734 (Sharova N.Yu., Nikiforova T.A., Pozdnyakova T.A.). The seed mycelium was grown on a medium containing (g/L) soy flour (10.0), glucose (10.0), NaCl (5.0), $CaCO_3$ (1.0), pH 7.0. The inoculate or seed material was added into a fermentation medium containing (g/L) cornstarch (20), soy flour (5.0), NaCl (3.0), KH_2PO_4 (1.0), and $MgSO_4 \times 7H_2O$ (0.5), pH 7.0. The cultures were cultivated in shake-flasks (750 mL) containing 100 mL of the medium at 28°C and 200 rpm for 96 h [11, 12].

The biomass was then removed by microfiltration through the filter element cartridge (Sartorius, Ger-

many) with a polyethersulfone membrane (300 μm) at a pressure of 0.2 MPa and 25°C, followed by filtration through a membrane with pores of 0.45/0.20 μm to remove residual producer cells.

The resultant solution after microfiltration was purified by ultrafiltration in a flow regime through polysulfone hollow fibers, which hold compounds with a molecular weight (MW) of 15 kDa under a pressure of 0.2 MPa at 25°C. We used a column (5 × 25 cm, Biotest, Russia) with a filtering surface area of the fibers of 0.2 m^2. The flow rate was 50 mL/min. The resultant purified solution was clarified with activated coal at 70°C (2 g of the coal per 100 g of the concentrate) under occasional stirring. The clarified concentrate was dried at 50°C.

Purification of the inhibitors was carried out by gel chromatography on a column with Sephadex G-25 (2.2 × 65 cm, Pharmacia, Shweden) using distilled water as eluent at a flow rate of 6 mL/h cm^2. The purification was confirmed by the repeated chromatography under the same conditions.

Lyophilization of fractions of the inhibitor was performed in a low-temperature box TV-2000 in ampules (~2 mL) with a layer height of the solution of 12 mm. The ampules containing the samples were frozen in the vertical position at −50°C for 20 h. The preparations were dried on a TF-50 setting (Hochvakuumtechnik, Germany) at a heating rate of 20°C/h.

The coloration of the solutions was evaluated by optical absorption at 360 nm (D_{360}) on a SP-46 spectrophotometer (LOMO, Russia) in a 1-cm cuvette.

The protein content was determined by the Lowry method.

The composition of the inhibitors was studied by acidic hydrolysis with 2 n sulfuric acid at 100°C for 15, 30, 45, 60 min and 6 h in sealed glass ampules, followed by TLC and quantitative evaluation of carbohydrates and reducing sugars by the anthrone and Somogyi-Nelson methds [14].

TLC was performed on Sorbfil PTLC-P-C plates (10 × 10 cm) covered with CTX-1BE silica gel (8–12 μm) on a polymer support (IMID, Russia) in a system of n-butanol-acidic acid-water (3 : 1 : 2). The compounds were visualized with specific reactants for different sugar groups [14].

The IR spectra of the inhibitors were recorded on a Specord 75 K spectrometer (Germany) in the transmission regime (resolution, 4000; intensification, 8.0; velocity of the mirror, 0.6329; diafrgam, 100.00; DTGS detector, KBr; splitter, KBr).

The MW of the inhibitor was determined by gel filtration on columns (2.2 × 65 cm) with Sephadex G-25 (Pharmacia, Shweden) using N-α-benzoyl-arginine (MW 439.95; Sigma-Aldrich GmbH, Germany), NADP-Na (MW 765.4 Sigma-Aldrich GmbH, Germany), vitamine B_{12} (MW 1579.6; Verofarm, Russia), and polyethyleneoxide (MW 2000, RusHimtreyd, Russia) as markers and distilled water as eluent. The flow rate was 6 mL/h cm^2.

The pH stability of 0.1% solution of the inhibitor was studied in 0.1 M universal buffer (pH 2–12) in a thermostate for 3 h at 25 ± 1°C, followed by evaluation of the inhibitor activity.

Temperature stability was studied, keeping the 0.1% inhibitor solution in distilled water in a range of temperatures starting at −25°C for not less than 3 h, followed by evaluation of the inhibitor activity. The melting temperature was determined on a KSP1D instrument (Kruess, Germany).

The affinity of the inhibitors to organic solvents (chemically pure) was evaluated by dissolving the lyophilized preparations in anhydrous methanol (Tianjin Ruifengtiantai Internationa Trade, China), 96% ethanol (Rosbio, Russia), dimethylsulfoxide, n-butanol, chloroform (DMSO, Component-Reactiv, Russia), and acetone (Russia). These solutions were stirred for 10 min, followed by evaluation of the inhibitor activity.

The inhibitory activity was evaluated relative to porcine pancreatic α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanhydrolase) using pancreatin (Sigma-Aldrich, United Ststes, 4 × USP specification). The enzyme and inhibitors solutions were diluted with 0.01 M phosphate buffer, pH 7.0 and introduced in test tubes (0.5 mL), followed by the addition of the pancreatin solution (2 mg/mL). The solutions were quickly mixed and kept for 10 min at 37°C, followed by the addition of 1% soluble starch (2 mL) in 0.1 M phosphate buffer. The mixture was stirred and kept for 10 min at 37°C. The reaction was stopped by the addition of 0.1 M HCl (3 mL). Aliquotes of the reaction mixture (0.2 mL) were transferred into the other test tubes, followed by adding 0.01 M aqueous iodine solution (9.8 mL) and mixing. The solutions turned violet to different intensities depending on the amount of nonhydrolyzed starch. Immediately after mixing, the optical absorption values were measured on an SP-46 spectrophotometer (LOMO, Russia) at 660 nm in 10-cm cuvette, with distilled water as the comparison solution. Similarly, the control reactions were carried out using distilled water instead of the inhibitor and pancreatine solution (control 1) and the inhibitor solution (control 2). Control samples 1 and 2 became blue and pale violet, respectively.

The inhibitory activity unit was considered as the amount of the inhibitor that suppresses the activity of pancreatic α-amylase by 50% at 37°C and pH 7.0 for 10 min. The inhibitory activity (IA) and inhibitory units (IU) per mL or mg were calculated by the formula

$$IA = \frac{(D_{oo.} - D_2) \times 100}{(D_1 - D_2) \times 50} K,$$

where $D_{oo.}$ is the optical absorption of the experimental sample, nm; D_1 is the optical absorption of the control sample 1, nm; D_2 is the optical absorption of the

control sample 2, nm; $\frac{(D_{oo.} - D_2)}{(D_1 - D_2)} \cdot 100$ is the extent of inhibition, %; K is the dilution factor of the experimental sample; and 50 is the coefficient of the calculation of the inhibitory extent by 50%. The extent of inhibition should be in the range of 40–55%.

We studied the effect of the inhibitors on α-amylase from *Bacillus subtilis* (Sigma, United States), α-amylase from *Aspergillus niger* (Shandong Longda Bio-Products Co, China), and human α-amylase. The human amylase was isolated from blood serum purified on a Cogent μScale setting for tangential filtration with a set of Pellicon XL cassettes (50–150 cm^2, (Merck Millipore, Germany). Compounds with a molecular weight from 1000 to 5 kDa were successively retained in the membrane at a filtration flow rate of 17 mL/min, pressure of 0.55 MPa, and 4°. Fine purification was carried out by affinity chromatography on a column (2.2 × 65 cm) with native starch equilibrated with 0.05 M Tris-HCl buffer, pH 6.5, with the same buffer containing 0.4% glycogen as eluent. The flow rate was 10 mL/h cm. Interaction with glucoamylase (EC 3.2.1.3; 1,4-α-D-glucan glucohydrolase) from *Aspergillus niger* (Shandong Longda Bio-Products Co., China) was studied according to [15]. The results were compared with the glucosidase inhibitor acarbose, the active substance of the antidiabetic preparation glucobay. Acarbose was extracted from glucobay (Bayer Pharma AG, Germany) (100 mg of the active substance, 2 mL of distilled water per one tablet) for 1 h at 25°C. The acarbose separated from the water-insoluble excipients andpurified by gel filtration on a column (2.2 × 65 cm) with Sephadex G-25 (Pharmacia, Sweden) with distilled water as an eluent at a flow rate of 6 mL/h cm^2, and lyophilized under the above conditions. The inhibitory activity of the resultant acarbose preparation was evaluated with respect to α-amylase from porcine pancreas (Sigma, United States).

The inhibition constants K_{in} and the inhibition type were determined by the graphical method [16].

RESULTS AND DISCUSSION

The total yield of the inhibitor after purification of the native solution according to the above scheme was 65 ± 7%. The content of pigments and protein impurities were decreased by three and ten times, respectively (Table 1).

Using quantitative methods for determining sugars, we found that the inhibitors contained 86 ± 2% compounds of a carbohydrate nature.

The preparations retained almost 100% activity under changing temperature and pH values. The properties did not change over a prolonged period of time (>12 months). The results are important for the production, storage, and use of these substances.

The purification of the obtained preparations by gel-chromatography resulted in two fractions with inhibitory activity (Table 2). The elution profile during rechromatography of each fraction showed one peak, which indicated the high purity of the inhibitors.

After lyophilization of each fraction, the yield of the inhibitor was 1–1.2 g from 1 L of the native solution. The results are comparable to the yields of inhibitors prepared by the conventional techniques of purification of the native solutions and the isolation of these inhibitors from these solutions [1].

The activity of inhibitor components from *S. lucensis* VKPM Ac-1743 were (350 ± 20) IU/mg (component 1) and (250 ± 10) IU/mg (component 2). The activity of the inhibitor components from *S. violaceus* VKPM Ac-1734 were (300 ± 20) IU/mg (component 1) and (200 ± 20) IU/mg (component 2).

The inhibitory activity of the isolated components s retained over a wide range of temperature and pH values (Table 2). As compared with the acarbose and the inhibitor from the *Streptomyces* sp. VKM 1328-D, the studied components are more resistant to elevated temperatures. The isolated inhibitors lose 7–9% of their activity at pH > 8. They have a high affinity to polar solvents and were low soluble in n-butanol and insoluble in chloroform. Components 1 have a similar mobility in an n-butanol-acidic acid-water system (3 : 1 : 2).

The MW values of components 1 exceeded those of components 2 by factors of 1.9–2. Multicomposition of the isolated inhibitors and their MW values are in an agreement with literature data.

The results of acidic hydrolysis showed that the content of reducing sugars in hydrolysates increased with increasing duration of the hydrolysis. After acidic hydrolysis for 6 h, components 1 and 2 contained 65% and 55% of reducing sugars, respectively. The TLC of hydrolysates showed the presence of aldose, the mobility of which was similar to glucose. No ketoses were revealed by TLC.

Quantitative analysis of the acidic hydrolysates showed that components 1 contained 6–7 glucose residues each, while acarbose and the inhibitor from *Streptomyces* sp. VKM 1328-D contained 2 and 8–9 glucose residues, respectively [1, 2, 17].

The IR spectra of the components of the inhibitors from *S. lucensis* VKPM Ac-1743 and *S. violaceus* VKPM Ac-1734 contained intensive bands character-

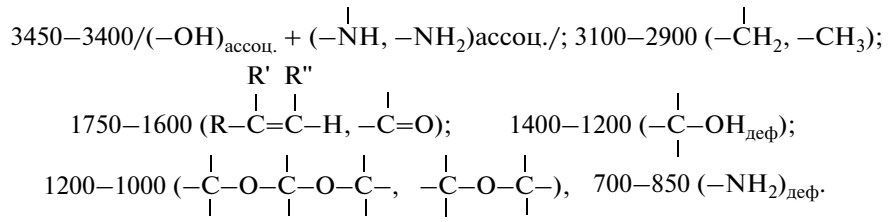
Table 1. Comparative characteristics of the isolation process from native solutions of glucosidase inhibitors synthesized by streptomycetes strains

Stages	Volume, mg; weight, mg	Inhibitory activity		Protein content		Coloration		Yield, %
		IU/mL, IU/mg	total IU × 10^5	mg/mL, mg/mg	purification factor	D_{360}	clarification factor	
Microfiltration								
S. lucensis VKPM Ac-1743	1000	37 ± 1	3700 ± 100	5.1 ± 0.5	–	8.3± 0.5	–	–
S. violaceus VKPM Ac-1734	1000	35 ± 2	3500 ± 200	3.8 ± 0.2	–	11.0 ± 0.5	–	–
Ultrafiltration								
S. lucensis VKPM Ac-1743	920 ± 20	3700 ± 100	34 ± 1	2.5 ± 0.1	2.0 ± 0.2	2.8 ± 0.1	3.0 ± 0.3	92 ± 2
S. violaceus VKPM Ac-1734	900 ± 10	3500 ± 200	32± 1	1.9 ± 0.1	2.0 ± 0.2	4.0 ± 0.1	2.8 ± 0.2	90 ± 2
Depigmentation								
S. lucensis VKPM Ac-1743	730 ± 20	3700 ± 100	27 ± 1	0.30 ± 0.01	8.3 ± 0.7	0.25 ± 0.01	11.2 ± 0.8	80 ± 2
S. violaceus VKPM Ac-1734	770 ± 20	3500 ± 200	27 ± 1	0.19 ± 0.01	10.0 ± 0.5	0.31 ± 0.01	12.9 ± 0.8	85 ± 2
Drying								
S. lucensis VKPM Ac-1743	4400 ± 200	500 ± 30	22 ± 1	0.050 ± 0.001	6.0 ± 0.3	–	–	82 ± 1
S. violaceus VKPM Ac-1734	5800 ± 250	400 ± 30	23 ± 1	0.030 ± 0.001	6.3 ± 0.6	–	–	86 ± 2

Table 2. Properties of α-glucosidase inhibitors

Characteristic	Inhibitor from *S. lucensis* VKPM Ac-1743	Inhibitor from *S. violaceus* VKPM Ac-1734	Acarbose [2]	Inhibitor from *S. species* VKM 1328-D [1]
Thermostability. °C	20–200	20–200	25–50	25–70
pH stability	2–8	2–8	1–12	1–12
Solubility	Soluble in water, ethanol, acetone; low soluble in DMSO, butanol; insoluble in chloroform	Soluble in water, ethanol, acetone; low soluble in DMSO, butanol; insoluble in chloroform	Soluble in water; insoluble in ethanol	Soluble in water, ethanol (acidic medium), DMSO, butanol, acetone; insoluble in cooled ethanol, chloroform
Mobility in an n-butanol-acidic acid-water system of (3 : 1 : 2), R_f:				
component 1	0.15	0.14	–	0.13
component 2	0.11	0.09	–	–
Melting temperature, °C				
component 1	145–147	144–146	–	192–194
component 2	133–135	128–129	–	–
Relative MW:				
component 1	2100–2300	1800–2000	645.61	2100
component 2	1100–1200	900–1000	–	–

istic of stretching and deformation vibrations and vibrations of the double bonds. The vibrations of the following groups were observed (ν, cm^{-1}):



Deformation and skeletal vibrations of polyatomic systems are in the region of the spectrum below 1500 cm^{-1}. This region contained bands characteristic to α-1,4- and α-1,2 glucoside bonds (ν = 934, 938, and 756 cm^{-1}). The presence of these bonds was confirmed by studying the interaction of the isolated inhibitors with glucoamylase, which catalyzed hydrolysis of these bonds in substrates at their concentrations of more than 0.01 g/mL in the reaction mixture. The K_M value was $(1.2 \pm 0.1) \times 10^{-2}$ M for components 2.

The above data indicate the carbohydrate nature of the isolated inhibitors.

The isolated inhibitors significantly repressed the activity of glucosidase of various origins when hydrolyzing starch (Table 3). Along with α-amylases, they inactivated glucoamylase, which catalyzed the hydrolysis of not only starch but also oligo- and disaccharides [18]. All components inhibited bacterial α-amylase to the greatest extent and exhibited a high affinity to amylase from human blood. The inhibitors from *S. violaceus* VKPM Ac-1734 repressed pancreatic α-amylase to a greater extent than inhibitors from *S. lucensis* VKPM Ac-1743, i.e. the K_{in} value of the latter was higher by an order of magnitude. The effect of components 1 and 2 from *S. lucensis* VKPM Ac-1743 and *S. violaceus* VKPM Ac-1734 on fungal amylases was different. In all systems, the inhibition of enzymes was reversible and competitive. Unlike acarbose and the inhibitor from *Streptomyces* sp. VKM 1328-D, the studied inhibitors exhibited an affinity to bacterial amylase.

The isolated inhibitors belong to the pseudooligosaccharides. They consist of two structural forms, have pronounced hydrophilicity, and are resistant to changes in temperature and pH. Independent of the strain, components 1 and 2 have similar MW values. The inhibitors can repress the activity of enzymes of different origin. They competitively inhibit the activity of both α-amylase and glucoamylase. This indicates that the inhibitors compete with substrates for binding to the active centers of enzymes. The identified com-

Table 3. Action of inhibitors synthesized by streptomycetes strains on αglucosidase of different origin

Enzyme	K_{in}, M					
	inhibitor from *S. lucensis* VKPM Ac-1743		inhibitor from *S. violaceus* VKPM Ac-1734		acarbose [19, 20]	inhibitor from *S. species* VKM 1328-D [1, 21]
	component 1	component 2	component 1	component 2		
Pancreatic α-amylase	$(6.5 \pm 0.2) \times 10^{-5}$	$(1.5 \pm 0.1) \times 10^{-5}$	$(4.3 \pm 0.2) \times 10^{-6}$	$(6.7 \pm 0.2) \times 10^{-6}$	7.9×10^{-7}	1.6×10^{-8}
α-Amylase from *A. niger*	$(1.1 \pm 0.1) \times 10^{-6}$	$(5.8 \pm 0.1) \times 10^{-5}$	$(2.3 \pm 0.1) \times 10^{-6}$	$(8.2 \pm 0.1) \times 10^{-5}$	–	–
α-Amylase *B. subtilis*	$(4.8 \pm 0.2) \times 10^{-8}$	$(6.3 \pm 0.2) \times 10^{-8}$	$(1.8 \pm 0.1) \times 10^{-8}$	$(8.3 \pm 0.2) \times 10^{-8}$	1.3×10^{-5}	9.4×10^{-6}
α-Amylase from human blood	$(1.7 \pm 0.1) \times 10^{-6}$	$(2.2 \pm 0.1) \times 10^{-6}$	$(1.9 \pm 0.1) \times 10^{-6}$	$(3.5 \pm 0.1) \times 10^{-6}$	–	–
Glucoamylase from *A. niger*	$(3.4 \pm 0.1) \times 10^{-6}$	$(4.9 \pm 0.1) \times 10^{-5}$	$(8.3 \pm 0.1) \times 10^{-7}$	$(2.4 \pm 0.1) \times 10^{-6}$	10^{-12}	3.7×10^{-7}

petitive type of inhibition implies a similarity in the inhibitor and substrate structure. The soluble starch used in the experiments as a substrate contains oligosaccharides. As established by acid hydrolysis and IR spectroscopy, the studied inhibitors contain 3–7 glucose residues each. This indirectly indicates a similarity between the inhibitor and substrate structure.

Comparison of the results with the characteristics of acarbose showed that the isolated inhibitors had larger MW values, contained more carbohydrate residues, and differed in physic-chemical and biochemical properties. At certain concentrations, they were substrates for glucoamylase, which is not typical for inhibitory pseudomonosaccharides such as nojiremycin, deoxynojiremycin, valienamine, and validamine [2, 22]. Acarbose is widely used in medicine for the treatment of disorders of carbohydrate metabolism [2, 7, 23]. Due to its carbohydrate nature, it is used in conjunction with diet. In light of this, it is necessary to perform a detailed study of the isolated inhibitors as biologically active substances of the prophylactic or functional action.

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