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ROLE OF GLYCOSYLATION IN SECRETION AND STABILITY OF MICROMYCETES α-GALACTOSIDASE

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The effect of the glycosylation inhibitors (tunicamycin and 2-deoxy-D-glucose) on the activity, stability and production of fungal glycosidases has been studied. It was shown that inhibition of N-glycosylation sites did not affect the secretion of Aspergillus niger α-galactosidase, however reduced yield of Cladosporium cladosporioides and Penicillium canescens α-galactosidases. Changes in the level of O-glycosylation resulted in a significant reduction in the activity and stability of α-galactosidases of all three producers tested. Activity of the modified enzymes was significantly lower than that of the native ones, and was 2.6 and 0.33 U/mg for A. niger α-galactosidase, 3.3 and 32.5 U/mg for C. cladosporioides α-galactosidase, 11.66 and 31.1 U/mg for P. canescens α-galactosidase, respectively. A. niger α-galactosidase completely lost activity during purification and storage. The decrease of thermal stability at 55 °C by 20% was shown for C. cladosporioides and P. canescens α-galactosidases. It was also noted that O-deglycosylation led to a decrease in resistance of these enzymes to the action of proteases.

Key words: α-galactosidase, Aspergillus niger, Cladosporium cladosporioides, Penicillium canescens, tunicamycin, 2-deoxy-D-glucose, deglycosylation.

To date rather voluminous material has been accumulated in respect of the role of carbohydrate component in stability of glycoproteins. The number of N- and O-glycosylation sites per one protein molecule may be significantly distinct among enzymes of one group, may vary for enzymes from different producers and for the ones received under different conditions of growing the same biosynthetic as well. It is known [1] that carbohydrate component plays a substantial role in formation of quaternary structure of protein; it is responsible for protection from degrading influence of proteolytic enzymes during protein synthesis. The presence of N-glycosylated sites in polypeptide may determine the stability of enzyme and create a possibility for creation of supramolecular structures [2, 3]. It is shown [2] that N-bound carbohydrates play an important role in secretion, because they are mainly revealed in extracellular enzymes as a part of linker site of a molecule. O-glycosylation prevents accumulation of molecules of substrate in binding centers of enzymes and provides their stechiometric binding. The enzymes containing high percentage of carbohydrates (more than 10%) in molecular composition are, as a rule, distinguished for their resistance to the action of high temperatures and other factors of aggressive environment [4].

Earlier [5, 6] we isolated and purified to homogenous state by the methods of gel- and ion-exchange chromatography preparations of α-galactosidases of three micromycetes *Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium canescens*, determined their amino acid and monosaccharide composition, studied their physicochemical, catalytic properties, showed high stability during storage and also under changing conditions of reaction medium. The degree of glycosylation of α-galactosidases of *A. niger*, *C. cladosporioides*, *P. canescens* was 10.0, 10.0 and 16.5%, respectively. Since the character of glycosylation (N-, O- or mixed types) of these proteins is unknown, the aim of this research was to study the way how inhibitors of N- and O-glycosylation influence

the secretion and stability of *A. niger*, *C. cladosporioides* and *P. canescens* α-galactosidases.

Materials and Methods

The cultures of *A. niger*, *C. cladosporioides* and *P. canescens* were grown on the medium of such composition (g/l): soy flour – 15.0; urea – 0.3; $(NH_4)_2SO_4$ – 1.4; KH_2PO_4 – 2.0; $MgSO_4 \times 7H_2O$ – 0.3; $CaCl_2$ – 0.3; yeast autolysate – 0.5; pH 5.2. Cultivation was performed under submerged conditions in Erlenmeyer flask (500 ml) during 96 h at 25 ºC and the rate of shaker rotation 220 turns/min.

Preparations of α-galactosidases (native and modified) were obtained from supernatant of culture liquid of *A. niger*, *C. cladosporioides*, *P. canescens*, purified to homogenous state, that was confirmed by SDS-PAGE [5, 6].

The experiments on impact of inhibitors on α-galactosidases secretion were performed in the following way: culture of micromycetes was preliminarily grown during 48 h; mycelium was separated by centrifugation and put in the medium of standard composition containing a necessary inhibitor. The growing was performed in the presence of 2-deoxy-D-glucose in concentration of 100 and 150 µg/ml or tunicamycin – 15 µg/ml. Concentration of tunicamycin was chosen by experimental way; mycelium growth decrease was observed at high concentrations. Cultures have been grown further during 3 days. α-Galactosidase activity was determined with usage of *p*-nitrophenyl- α-D-galactopyranoside [7]. Protein content was measured by Lowry method [8], using as a standard bovine serum albumin. Total quantity of carbohydrates was determined by Dubois method [9].

Thermoinactivation of α-galactosidase was investigated at 55-60 °C, pH 5.2 (0.1 M phosphate-citrate buffer (PCB)). Samples of native and modified enzyme 0.05–0.5 U/ml in 0.1 M PCB, pH 5.2, was maintained at the set temperature during 1.5-3 h. At stated intervals (10-30 min) aliquots (0.1 ml) were selected and α-galactosidase activity was measured.

Such commercial preparations of proteolytic enzymes were used: proteinase K, pronase E, collagenase, trypsin (Merk, Germany), protosubtilin (Enzyme,Ukraine). The treatment of α-galactosidases preparations by proteases was performed in standard conditions in 0.1 M PCB pH 5.2 at temperatures 20 and 55 ºC. The ratio of galactosidase : protease was 1 : 1 (0.2 mg of enzymes in reactive solution).

Monosaccharide composition was studied after hydrolysis of enzymes preparations in 2 N HCl during 5 h at 100 ºC. The analysis of samples in the form of polyol acetates [10] was conducted by the method of chromato-mass-spectrometry using Agilent 6890N/5973 (USA), column DB-225 mS 30 m×0.25 mm×0.25 µm, gas-medium – helium, stream velocity through the column was 1 ml/min. The temperature of evaporator – 250 °C, interface – 280 °C, thermostate – 220 °C (isothermal regime). The sample was injected by separation of the stream 1:100. Monosaccharide identification was performed by comparison of the time of retaining polyol acetates of the standard and investigated samples and also with usage of computer database ChemStation. Quantitative content of individual monosaccharides was presented in percents of the total sum of squares of monosaccharide peaks [11].

Maximal reaction rate (V_{max}) and Michaels constant (K_m) were determined by the method of Lainuiver-Berk [12].

All the experiments were repeated no less than 3-5 times. Statistical treatment of experiments was performed by the standard method with usage of Student's *t*-criterion at 5% level. The results presented graphically were obtained with assistance of Microsoft Excel 2003 program. The values at $P < 0.05$ were considered as reliable.

Results and Discussion

The influence of glycosylation inhibitors on secretion and properties of proteins were studied before on insects, mushrooms and yeasts [2, 4, 13-15]. Tunicamicyn was most often used as an inhibitor of glycosylation. It prevents the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine on dolicholphosphate, thus uncoupling a metabolic way of N-glycosylation [16, 17]. Specific inhibitors of O-type glycosylation were not found up to date. 2-Deoxy-D-glucose plays this role most frequently. It is able under specific conditions to inhibit the reaction of interconversion of glucose-6-phosphate/mannose-6-phosphate [13]. This, in its turn, partially prevents formation of glycosylic links of O-type in glycoconjugates.

To study the role of N- and O-glycosylation in secretion and stabilization of α-galactosidases of *A. niger*, *C. cladosporioides*, *P. canescens* tunicamicyn and 2-deoxy-D-glucose were used as inhibitors respectively; cultures of micromycetes were grown on nutrient medium in the presence of these inhibi-

tors. Preparations of *A. niger*, *C. cladosporioides*, *P. canescens* α-galactosidases were obtained from supernatants of cultural liquids of producers grown under ordinary conditions and in the presence of inhibitors of glycosylation. They were purified by the methods of chromatography.

The influence of tunicamycin in concentration of 15 μg/ml was investigated, since high concentrations (more than 50 μg/ml) may cause a decrease of mycelium growth. It is shown (Table 1) that in this case some decrease of α-galactosydase activity is observed in cultural liquid of all three micromycetes. However, a decrease of general secretory protein was marked only in *C. cladosporioides* and *P. canescens*. Calculations of Michaels constant K_m for modified enzymes showed that affinity of *A. niger* α-galactosidase for nitrophenyl substrate did not change (K_m of initial preparation was 1.10 ± 0.02 mM, and modified one – 1.09 ± 0.02 mM). There was not an increase of K_m for enzymes of *P. canescens* (0.79 ± 0.03 mM) and *C. cladosporioides* (0.70 ± 0.01 mM) as compared with native enzymes (K_m 1.25 ± 0.01 and 0.90 ± 0.01 mM, respectively). It is necessary to note that indexes of specific activity for investigated α-galactosidases decrease (2 times for *A. niger*, 4.5 times for *P. canescens* and by 10% for *C. cladosporioides*). It can testify the important role of N-type of glycolysation of enzymes both in the display of glycosydases' activity and in their secretion. However, it should be noted that we do not observe a decrease of thermostability (55-60 ºC) and resistance to proteolysis of glycosidases of *P. canescens* and *C. cladosporioides* modified by tunicamycin. This also may testify that a decrease of N-glycosylation first of all influence the change of the level of these enzymes in cultural liquid, but not the stability under conditions of thermal denaturation.

It can be assumed, that secretion of *A. niger* α-galactosidase either does not depend on the level of N-glycosylation of protein molecule or, that the secretion decrease under the conditions of experiment was so insignificant that entered the range of the experiment error. The diminishing of the enzyme half-life period at 55-60 ºC from 70 min to 30 min was observed.

The availability of N-bound carbohydrates in molecules of all three α-galactosidases is proved by the data of monosaccharide content of native enzyme preparations. Thus, earlier [5, 6] it was determined that carbohydrate part of *A. niger* α-galactosidase was presented by mannose, galactose, D-glucosamine and by two unidentified amino sugars (10:2:1:1:1). These carbohydrates are present in all enzyme preparations tested. A decrease of mannose content by 5-70% was admitted as a result of studying the monosaccharide content of modified α-galactosidases preparations (Table 2).

There are data in the literature that the inhibition of glycosylation of N-type is not always accompanied by the decrease of exoenzymes level, thus the correlation between N-glycosylation and secretion level is not marked. The same situation is probably observed in our case.

It is known [18] that the use of 2-deoxy-D-glucose causes considerable inhibition of O-glycosylation of secretory proteins of micromycetes. Thus, under the inhibitor concentration of 80 μg/ml the

Table 1. Influence of tunicamycin and 2-deoxy-D-glucose on secretion of micromycetes α-galactosidases

Parameters	Content, % (control 100 %)		
	Tunicamycin, 15 μg/ml	2-deoxy-D-glucose, 100 μg/ml	2-deoxy-D-glucose, 150 μg/ml
α-Galactosidase of *A. niger*			
Total protein content	120 ± 5	110 ± 5	103 ± 6
Activity	97 ± 2	104 ± 4	86 ± 2
α-Galactosidase of *C. cladosporioides*			
Total protein content	83 ± 3	102 ± 5	106 ± 5
Activity	95 ± 5	140 ± 8	104 ± 5
α-Galactosidase of *P. canescens*			
Total protein content	85 ± 4	104 ± 5	102 ± 4
Activity	58 ± 3	98 ± 5	108 ± 5

T a b l e 2. Change of carbohydrate content in native and modified preparations of α-galactosidases

α-Galactosidase preparation	Carbohydrates (% of glycosylation, without of inhibitor – 100%)			
	Mannose	Galactose	Arabinose	Rhamnose
A. niger N-modified	28.5	44.0	–	–
A. niger O-modified	52.3	12.3	–	–
P. canescens N-modified	81.2	–	81.1	50.9
P. canescens O-modified	95.1	–	48.8	5.2
C. cladosporioides N-modified	94.5	–	120.1	94.2
C. cladosporioides O-modified	30.8	–	115.6	95.1

Foot-note: "-" – absent

O-glycosylation is repressed by 20% and under the inhibitor concentration of 160 μg/ml – by 40-55%. However, even at so significant changes in carbohydrate structure, a decrease of secretion level may not be observed. Such a decrease was not marked in our experiments (Table 1), except for the use of 150 μg/ml of 2-deoxy-D-glucose under cultivation of *A. niger*. However, in this case we have to do with the change of catalytic properties of enzyme as a result of loss of carbohydrate component, because in the process of purification and storage the activity of *A. niger* α-galactosidase was practically fully lost (0.02 U/ml).

Studies of monosaccharide composition and stability of purified α-galactosidases preparations, received as a result of micromycetes growing in the medium with 150 μg/ml of 2-deoxy-D-glucose, have shown that carbohydrate content in enzymes was as follows: from *A. niger* – 16 μg/ml, *C. cladosporioides* – 10 μg/ml, *P. canescens* – 28 μg/ml. Information on monosaccharide composition (Table 2) demonstrates the considerable diminishing of the amount of some carbohydrates in enzyme preparations. At the same time we marked a significant decrease of the activity of obtained preparations as compared with native ones. Thus, the activity of *A. niger* α-galactosidase was 0.33 U/mg, that is almost 9 times less than the activity of initial enzyme preparations (2.6 U/mg). Activities of modified *C. cladosporioides* (3.3 U/mg) and *P. canescens* (11.66 U/mg) α-galactosidases were also lower than activities of original enzymes (32.5 U/mg and 31.1 U/mg, respectively). The change of normal O-glycosylation often causes the lowering of thermo-, pH-stability, resistance to action of proteases that may identify conformational changes, while N-bound sugars are, first and foremost, the signals for providing of normal secretory process.

We have studied thermal stability of O-deglycosated α-galactosidase preparations at 55 ºC, pH 5.2. It is shown (Fig. 1) that in all cases a certain decrease of stability (up to 20%) was observed under incubation during 3 hours. It should be noted that *A. niger* α-galactosidase has completely lost its activity during 2 months of storage.

It was also shown that a decrease of carbohydrate content in molecules of *P. canescens* and *C. cladosporioides* α-galactosidases is accompanied by decline of resistance of these enzymes to proteases action under conditions of thermal denaturation (Fig. 2, 3). Thus, α-galactosidase of *P. canescens* showed a higher resistance to trypsin, collagenase and protosubtilin than to proteinase K and pronase E.

Thus, the conducted investigations have shown that the studied α-galactosideses are enzymes, which are glycosylated by mixed type. However, there were significant differences in the degree of carbohydrate influence on secretion and properties of α-galactosidases from different sources. N-deglycosylation did not influence the secretion of exoenzyme of *A. niger*, but decreased its thermal stability. The disturbance of normal N-glycosylation of *P. canescens* and *C. cladosporioides* α-galactosidases, on the contrary, resulted in a decrease of enzymes output, but at the same time, practically did not influence their catalytic properties. The use of 2-deoxy-D-glucose did not influence the output of extracellular enzymes, however, led to a significant decrease of glycosidases stability. Activity of *A. niger* α-galactosidase was fully lost in the process of purification, but *P. canescens* and *C. cladosporioides*

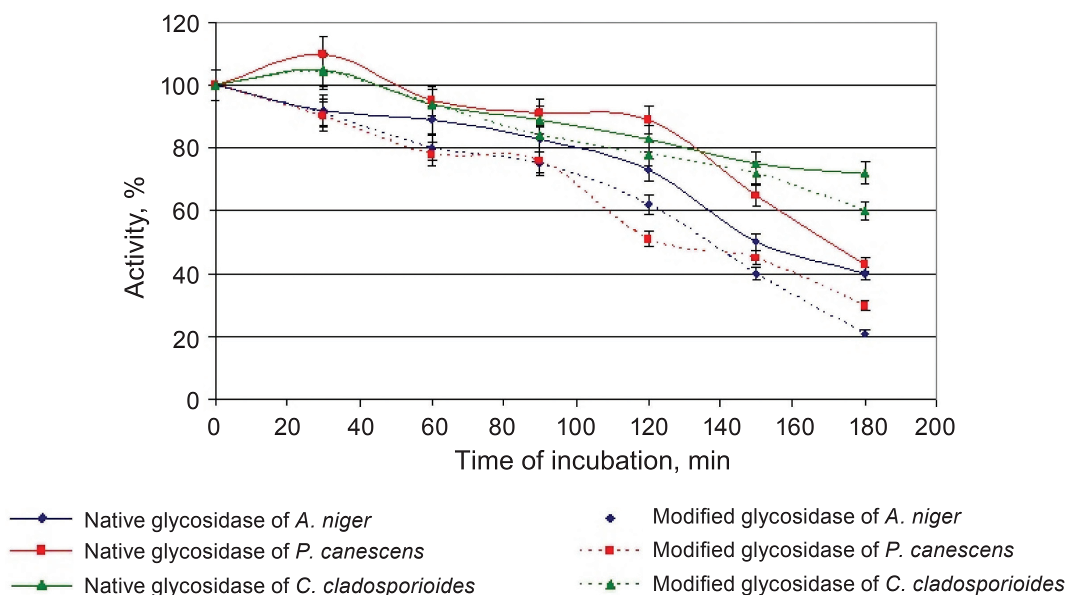

Fig. 1. Dependence of native and O-deglycosylated α-galactosidase of A. niger, P. canescens, C. cladosporioides on time during thermoinactivation (pH 5.2, 55 ºC)

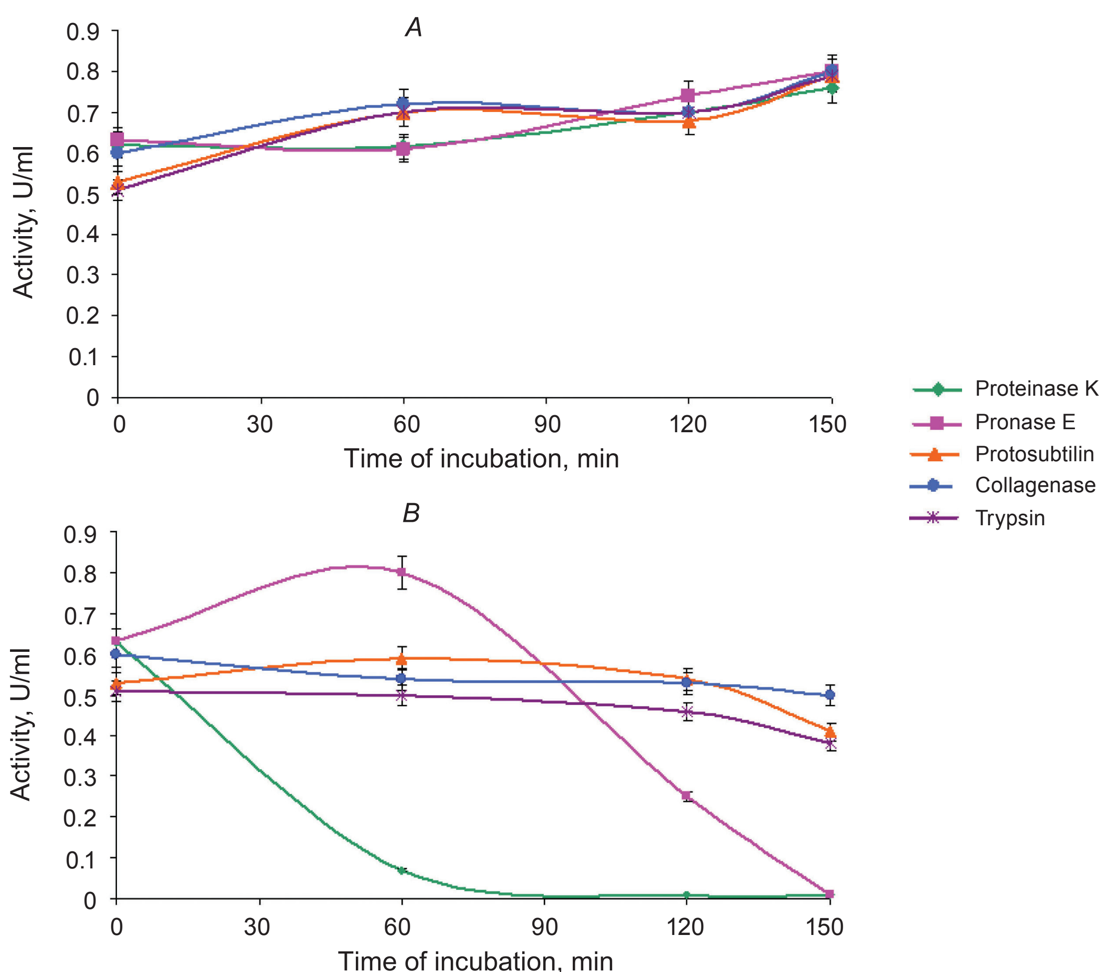

Fig. 2. Dependence of activity of P. canescens α-galactosidase on time during treatment by proteolytic enzymes (A – at 20 ºC, B – at 55 ºC)

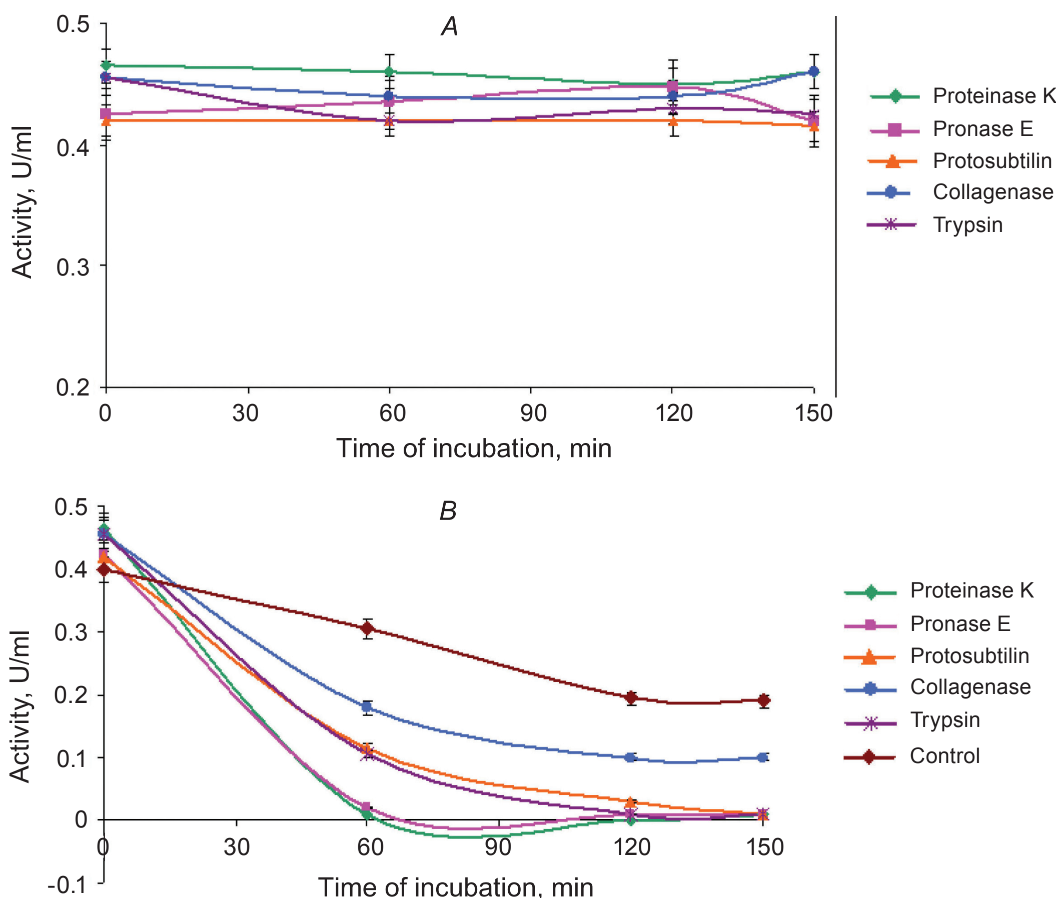

Fig. 3. Dependence of activity of C. cladosporioides α-galactosidase on time during treatment with proteolytic enzymess (A – at 20 °C, B – at 55 °C)

α-galactosidases demonstrated a marked decrease of resistance to the action of proteolytic enzymes and thermal denaturation.

РОЛЬ ГЛІКОЗИЛЮВАННЯ У СЕКРЕЦІЇ ТА СТАБІЛЬНОСТІ α-ГАЛАКТОЗИДАЗ МІКРОМІЦЕТІВ

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Досліджено вплив інгібіторів глікозилювання (тунікаміцину та 2-дезокси-D-глюкози) на активність, стабільність та продукцію глікозидаз мікроміцетів. Показано, що інгібування N-глікозилювання не впливало на секрецію α-галактозидази *Aspergillus niger*, проте знижувало вихід α-галактозидаз *Cladosporium cladosporioides* і *Penicillium canescens*. Зміна ступеня О-глікозилювання призводила до зниження активності та стабільності α-галактозидаз трьох продуцентів. Активність модифікованих ензимів була значно нижчою, ніж нативних, і становила відповідно 0,33 та 2,6 од./мг для α-галактозидази *A. niger*, 3,3 та 32,5 од./мг для α-галактозидази *C. cladosporioides*, 31,1 та 11,66 од./мг для α-галактозидази *P. canescens.* α-Галактозидаза *A. niger* повністю втрачала активність впродовж очистки та зберігання. Для α-галактозидаз *C. cladosporioides* і *P. canescens* показано зниження термостабільності до 20% при 55 °С. Також відмічено, що О-деглікозилювання знижувало стійкість цих ензимів до дії протеаз.

Ключові слова: α-галактозидаза *Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium canescens*, тунікаміцин, 2-дезокси-D-глюкоза, деглікозилювання.

РОЛЬ ГЛИКОЗИЛИРОВАНИЯ В СЕКРЕЦИИ И СТАБИЛЬНОСТИ α-ГАЛАКТОЗИДАЗ МИКРОМИЦЕТОВ

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Исследовано влияние ингибиторов гликозилирования (туникамицина и 2-дезокси-D-глюкозы) на активность, стабильность и продукцию гликозидаз микромицетов. Показано, что ингибирование N-гликозилирования не оказывало влияния на секрецию α-галактозидазы *Aspergillus niger*, однако снижало выход α-галактозидаз *Cladosporium cladosporioides* и *Penicillium canescens*. Изменение степени O-гликозилирования приводило к снижению активности и стабильности α-галактозидаз трех продуцентов. Активность модифицированных энзимов была значительно ниже, чем нативных, и составляла соответственно 0,33 и 2,6 ед./мг для α-галактозидазы *A. niger*, 3,3 и 32,5 ед./мг для α-галактозидазы *C. cladosporioides*, 11,66 и 31,1 ед./мг для α-галактозидазы *P. canescens*. α-Галактозидаза *A. niger* полностью инактивировалась в процессе очистки и хранения. Для α-галактозидаз *C. cladosporioides* и *P. canescens* показано снижение на 20% термостабильности при 55 ºС. Также отмечено, что O-дегликозилирование снижает устойчивость этих энзимов к действию протеаз.

Ключевые слова: *Aspergillus niger, Cladosporium cladosporioides, Penicillium canescens*, α-галактозидаза, туникамицин, 2-дезокси-D-глюкоза, дегликозилирование.

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