ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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STABILITY OF NATIVE AND MODIFIED α-GALACTOSIDASE OF *Cladosporium cladosporioides*

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By modifying carbohydrate component of glycoproteins it is possible to elucidate its role in manifestation of structural and functional properties of the enzyme. The comparison of activity and stability of the native and modified by oxidation with sodium periodate α-galactosidase of Cladosporium cladosporioides was carried out. To determine α-galactosidase activity the authors used n-nitrophenyl synthetic substrate, as well as melibiose, raffinose and stachyose. Modification of the carbohydrate component had a significant effect on catalytic properties of the enzyme. Both the reduction of V_{max} and enzyme affinity for natural and synthetic substrates were observed. The native enzyme retained more than 50% of the maximum activity in the range of 20-60 °C, while for the modified enzyme under the same conditions that temperature range was 30-50 °C. The modified α-galactosidase demonstrated a higher thermal stability under neutral pH conditions. The residual activity of the modified α-galactosidase was about 30% when treated with 70% (v/v) methanol, ethanol and propanol. About 50% of initial activity was observed when 40% ethanol and propanol, and 50% methanol were used. It was shown that the modification of C. cladosporioides α-galactosidase by sodium periodate is accompanied by a significant decrease in enzyme activity and stability, probably caused by topological changes in the tertiary and quaternary structure of the protein molecule.

K e y w o r d s: α-galactosidase, Cladosporium cladosporioides, sodium periodate, glycosylation, modification.

Glycosylation is one of the most important post-translational modifications, both integrated membrane and secretion proteins being subjected to it. Glycosylated proteins play an important role in bioregulation, transport, identification of biological micromolecules. The investigations of composition, synthesis and biological significance of glycoproteins cause great interest today, since they can help to establish the structure-function consequences of glycosylation and the role of carbohydrate component in the processes of protein functioning [1].

Proteins may be glycosylated following the pattern of N- and O-glycosylation. N-glycosylation is performed by asparagin distributed over one amino acid residue from tryptophan, and proceeds by stages. In the process of O-glycosylation one-two carbohydrate residues are mainly added to serine and threonine as well as to hydroxylysine and hydroxyproline. Mannose, galactose, glucose, xylose, fucose, arabinose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine are the most distributed monosaccharides being the components of glycoproteins; but this list has been considerably extended in the recent years [1, 2]. The number of sites of N- and O-glycosylation per one molecule may differ considerably among the enzymes of one group, differences in protein glycosylation degree are shown both for the enzymes from various producers and for those obtained under different conditions of growing one and the same biosynthetic. The presence of N-glycosylated sites in polypeptide may determine the enzyme stability and create a possibility of formation of supermolecular structures. N-bound carbohydrates play a significant role in secretion, since they are mainly found in extracellular enzymes in the composition of the molecule linker site. O-glycosylation prevents from accumulation of the substrate molecules in the binding centers of the enzyme and provides their stoichiometric binding. Highly glyco-

sylated enzymes are as a rule distinguished by resistance to the influence of high temperatures and other factors of the reaction medium [1].

We have earlier obtained α-galactosidase (EC 3.2.1.22) of *Cladosporium cladosporioides*, and it has been shown [2] that it is a high-molecular enzyme with Mw 400 kDa, consisting of 4 subunits. The enzyme has been glycosylated following the mixed type, the content of carbohydrates is 10% (m/m). Mannose and D-glucosamine have been identified in the composition of *C. cladosporioides* α-galactosidase, arabinose and rhamnose have also been found, and their amounts prevailed. In this connection the investigations of oxidation of such enzyme carbohydrate component are interesting both from the viewpoint of glycosylation effect on glycoprotein activity and stability and on its effect on formation of subunit interactions inside the enzyme molecule. Broad substrate specificity of *C. cladosporioides* α-galactosidase permits estimating the contribution of the carbohydrate component to formation of the enzyme-substrate complex.

Various approaches are used to study the role of carbohydrates in the display of biological activity of glycoproteins. One of the methods is chemical modification by means of oxidation with sodium periodate which favors the splitting of the pyranose ring with formation of C3, C4-aldehydes. This method is fit for nonselective effect on N- and O-bound oligosaccharides under the minimal influence on the polypeptide chain [3].

The work was aimed to obtain the α-galactosidase preparation oxidized by sodium periodate and to conduct comparative investigations of activity and stability of the native and modified glycosidase of *C. cladosporioides.*

Materials and Methods

α-Galactosidase preparation was obtained from the culture liquid of micromycetes *C. cladosporioides* (Museum of Live Cultures, D. K. Zabolotny Institute of Microbiology and Virology, NAS of Ukraine) and purified by gel filtration and ion exchange chromatography with Toyoperl HW-60, DEAE-Toyoperl 650s и Sepharose 6B according to already developed procedure [4].

α-Galactosidase activity was determined with the help of synthetic substrate – *n*-nitrophenyl-α-D-galactopyranoside (Sigma-Aldrich, USA) [5]. Protein was determined by the Lowry method [6], and serum albumin was used as a standard. The total amount of carbohydrates was determined with phenol and sulfuric acid [7].

α-Galactosidase activity, when using natural substrate of melibiose, raffinose and stachyose, was determined by the amount of formed galactose by the Somogyi-Nelson method.

Such amount of enzyme, which hydrolysed 1 μmol of substrate for 1 min in the experiment conditions, was taken as the activity unit.

Effect of pH and temperature was investigated in the range of pH 2.0-8.0, and temperature 20-70 °C.

Thermal inactivation of α-galactosidase was conducted at temperature 40 and 52 °C, pH 3.5, 5.0, 6.0 and 7.0, where 0.1 M phosphate-citrate buffer (PCB) was used. The kinetics of thermal inactivation was investigated as follows. The samples of native and modified enzyme 0.5 U/ml in 0.1 M of PCB, with corresponding pH, were kept at pre-set temperature during 3 h. Aliquots of 0.1 ml were taken every 10-30 min, and α-galasctosidase activity was measured.

Oxidation with periodate was conducted according to the following method [9]. The enzyme preparation (0.5 mg/ml of protein, 25 U/ml) in 0.1 M of PCB was treated by 0.5 ml of 10 mM solution of sodium periodate (Shanghai Synnad, China). The reaction mixture was incubated for 15 min in darkness. The oxidation process was stopped by adding 0.25 ml of ethylene glycol per 1 ml of the sample. Then the reaction mixture was subjected to gel filtration on Sepharose 6B (0.1 M PCB, pH 5.2).

The dependence of activity of native and modified *C. cladosporioides* α-galactosidase on concentration of *n*-nitrophenyl substrate (from 0.1 to 10.0 mg/ml) was determined in standard conditions (37 °C, pH 5.2).

The effect of alcohols (methanol, ethanol, propanol, glycerol) was studied in standard conditions (37 °C, pH 5.2) at various concentrations of reagents (0-80%) and enzyme concentration 2 mg/ml.

Maximum reaction rate (V_{max}) and Michaelis constant (K_m) were determined by Lineweaver-Burk method [10].

All experiments were repeated no less than 3-5 times. Statistical processing of the results of experimental series was performed by standard methods with the use of Student's *t*-criterion at 5% level of significance. The results were processed and presented as diagrams with the help of the program Microsoft Excel 2003. The values at $P < 0.05$ were considered as reliable.

Results and Discussion

The effect of glycosylation on thermodynamic and kinetic properties of proteins is poorly studied now. However, a significant role of the carbohydrate component in ensuring stability and in the processes of protein folding gains ever increasing corroboration in the works of recent years [11, 12]. And moreover, the works directed to increasing stability by chemical glycosylation of proteins receive more and more recognition [13]. At the same time, deglycosylation of proteins can result both in insignificant changes of their properties and in complete loss of functional activity. Oxidation with sodium periodate permitted obtaining a modified form of *C. cladosporioides* α-galactosidase with low content of carbohydrate part (the amount of carbohydrates decreased to 3.2%, m/m). The modification resulting in the decrease of carbohydrates content to 2% and below was accompanied by practically complete loss of α-galactosidase activity.

A decrease of such indices as maximum rate of substrate hydrolysis (V_{max}) and affinity for the substrate (K_m) was shown as a result of investigation of the substrate concentration effect on activity of native and modified α-galactosidase. The activity in respect of the synthetic substrate decreased from 23.8 U/mg to 3.9 U/mg of protein; a decrease of hydrolysis rate of melibiose, raffinose and stachyose was observed as well (Table). A simultaneous decrease of the enzyme affinity for nitrophenyl and natural substrates was noted. But as soon as the observed K_m differences were inconsiderable, one can suppose that the substrate-binding sites were not touched upon as a result of decreasing the glycosylation degree, and a decrease of catalytic activity is connected with conformational changes of a protein.

A decrease of activity and stability of the modified preparation was also noticed as a result of studying physico-chemical properties of the obtained *C. cladosporioides* α-galactosidase. Thermal optimum of the modified enzyme, as well as of the native one, is observed at 50 °C, but (Fig. 1) such a temperature range narrowing is observed on the background of the decrease of the enzyme activity, α-galactosidase activity being noted. The native enzyme holds more than 50% of maximal activity in the range of 20-60 °C, while for the modified enzyme this range is 30-50 °C under the same conditions. Investigations of pH-optimum of *C. cladosporioides* α-galactosidase have shown (Fig. 2) a certain shift in the range of activity of the modified enzyme from the acid to neutral zone.

Thermal stability of the native and modified enzymes was also studied under different pH values. The native (glycosylated) enzyme displayed a higher stability and activity in the experiment conditions. It was established (Fig. 3) that the resistance of enzymes to thermal denaturation was in direct dependence on the reaction medium pH. The modified enzyme displayed a higher stability in the neutral pH zone, while the native α-galactosidase activity was longer at acid pH values of the medium. These data can indirectly indicate that the number of sites of the enzyme glycosylation decreased as a result of treatment by sodium periodate. In accordance with the latest experimental data [11] the degree of thermal stabilization of protein depends, to a considerable extent, on the position and number of gly-

Kinetic parameters of the native and modified α-galactosidase of C. cladosporioides

Kinetic parameters	Native α-galactosidase	Modified α-galactosidase
V_{max}, μmol/min/mg of protein, pNPG	23.8 ± 0.5	3.9 ± 0.1*
K_m, mM, *p*NPG	0.90 ± 0.05	1.14 ± 0.05
V_{max}, μmol/min/mg of protein, melibiose	1.60 ± 0.05	0.80 ± 0.02*
K_m, mM, melibiose	1.70 ± 0.06	1.90 ± 0.06
V_{max}, μmol/min/mg of protein, raffinose	3.30 ± 0.08	2.70 ± 0.05*
K_m, mM, raffinose	2.20 ± 0.04	3.10 ± 0.07*
V_{max}, μmol/min/mg of protein, stachyose	2.50 ± 0.05	1.30 ± 0.03*
K_m, mM, stachyose	1.80 ± 0.03	2.50 ± 0.05*

Note: *$P < 0.05$

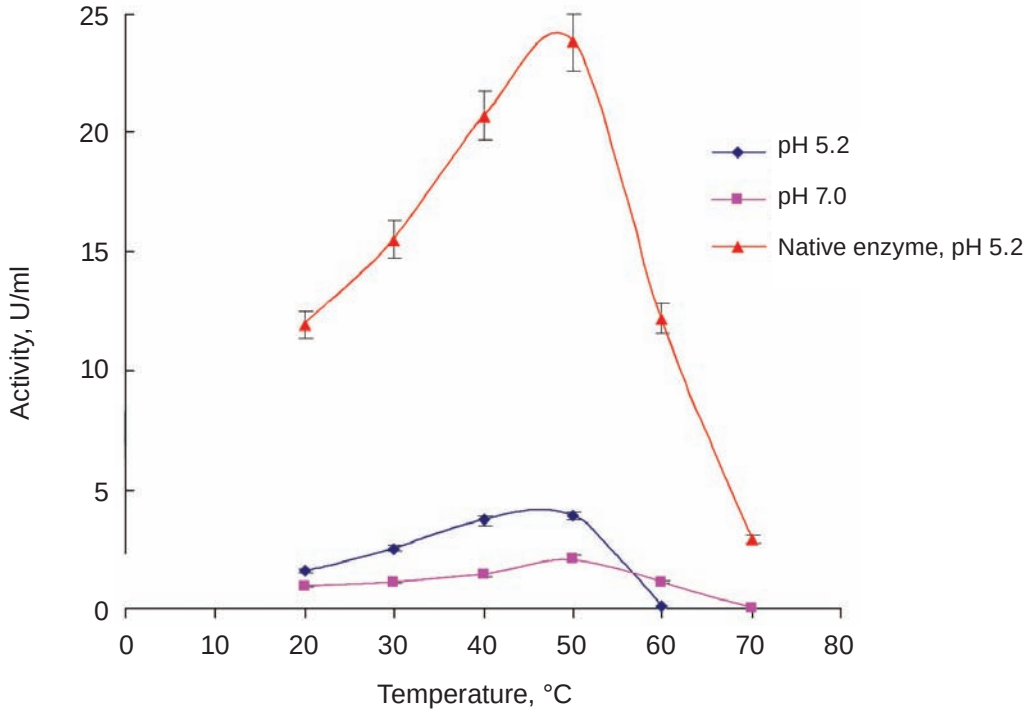

Fig. 1. Dependence of activity native and modified α-galactosidases of C. cladosporioides on the temperature (n = 5)

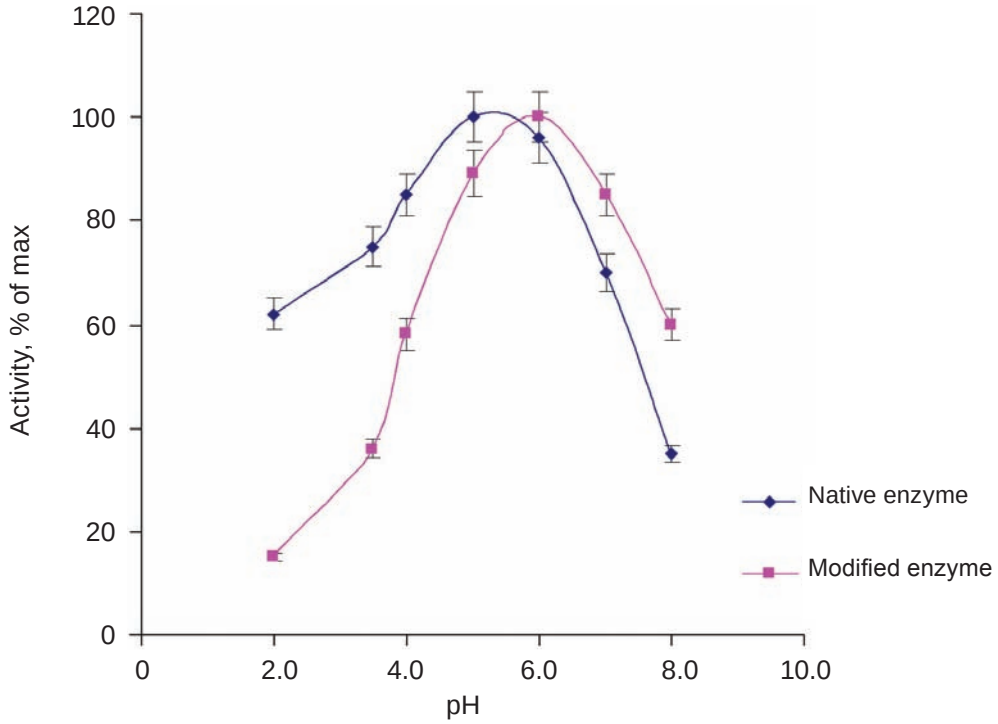

Fig. 2. Dependence of activity native and modified α-galactosidases of C. cladosporioides on pH, 37 °C (n = 5)

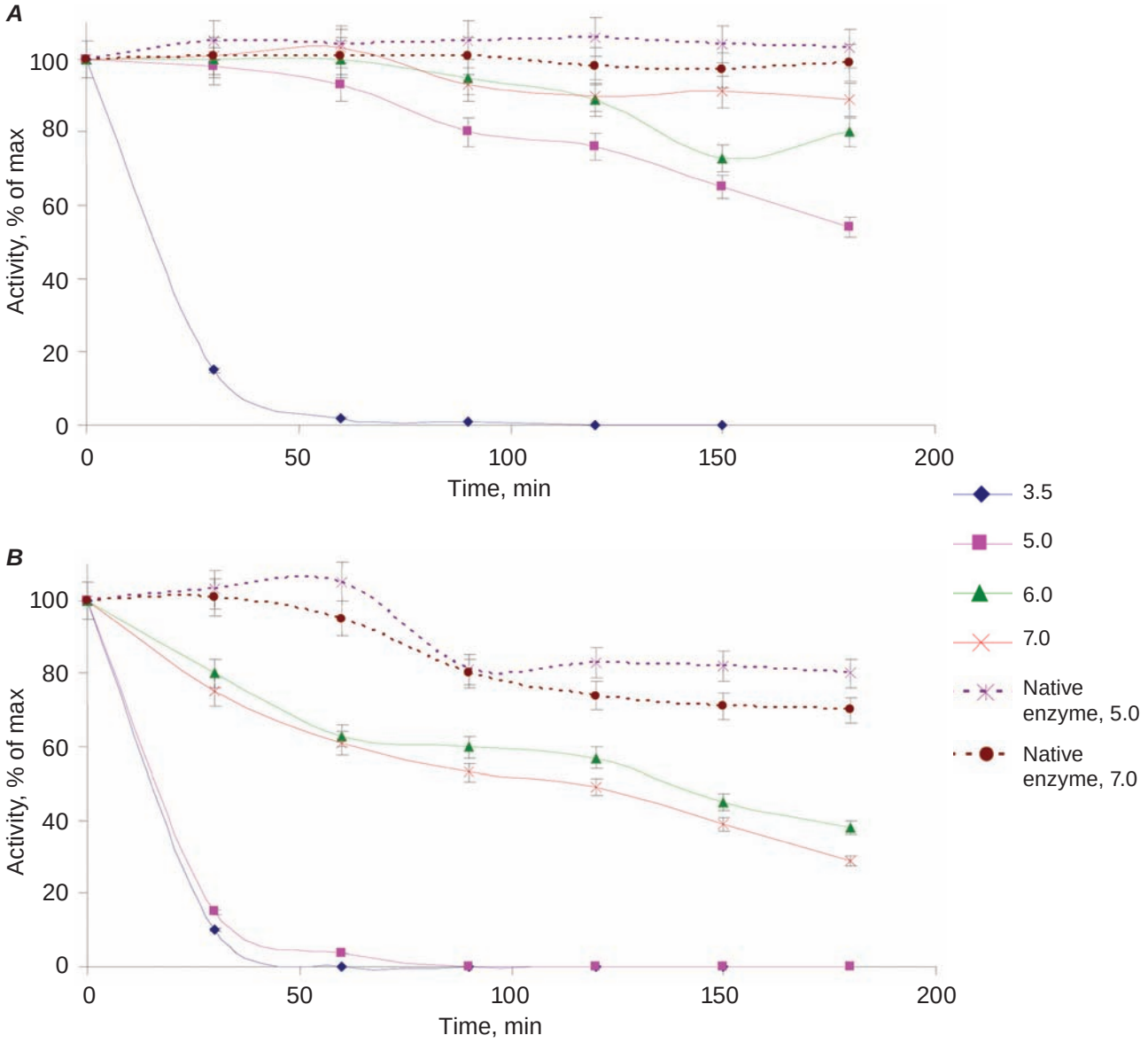

Fig. 3. Curves of thermal inactivation of the modified α-galactosidase of C. cladosporioides at different pH values, A - 40 °C, B - 52 °C (n = 3)

cosylation sites and is practically independent of the size of the attached glycan.

Investigations concerning the effect of alcohols on residual activity of two forms of *C. cladosporioides* α-galactosidase have shown (Fig. 4) that the enzyme displays a rather high resistance to the effect of organic solvents. Thus, about 30% of residual enzyme activity was noted under treatment with 70% (v/v) methanol, ethanol and propanol. About 50% of initial activity was recorded when using 40% ethanol and propanol, as well as 50% methanol. When analyzing the inactivation curves, one can make a conclusion that, all other things being the same, the inhibiting effect is intensified with the increase of the alcohol hydrocarbons chain length. A decrease in activity of the modified α-galactosidase in the presence of glycerol was also noted, while the protective effect of this reagent during 3 h (65 °C) was observed for the native enzyme [14]. This may point to the topological changes caused by the removal of glycoprotein carbohydrate component.

The value and contribution of the carbohydrate component to the display of biological activity of various glycoproteins is actively discussed in current literature [1, 9 11, 13]. The modulation of biophysical properties as a result of deglycosylation or,

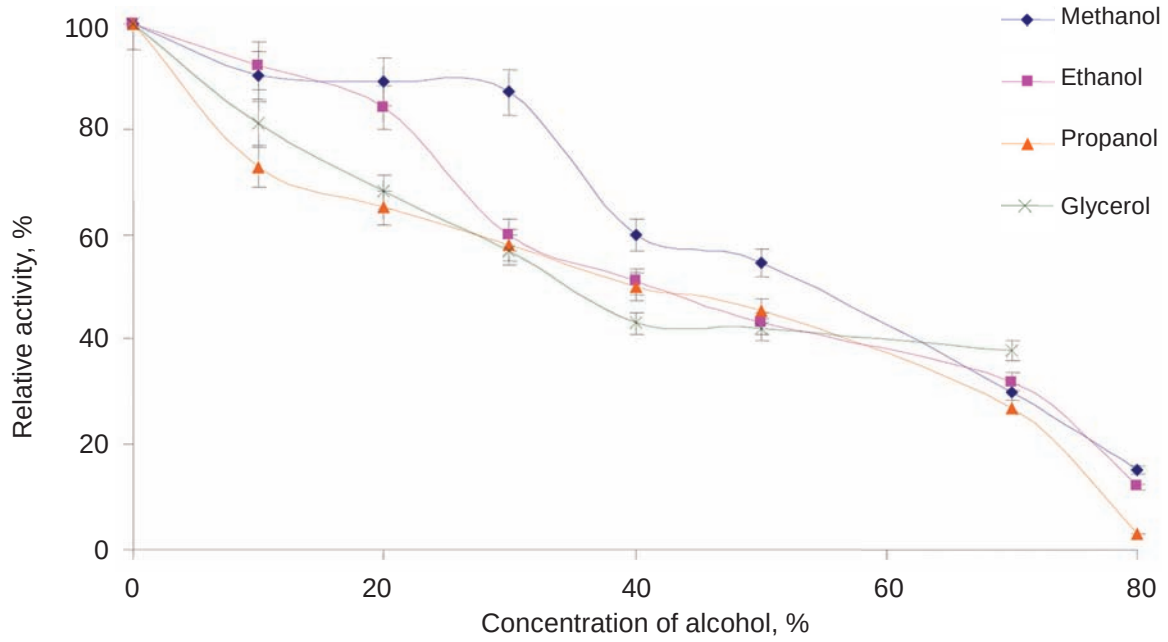

Fig. 4. Effect of alcohol concentration on the activity of the modified α-galactosidase of C. cladosporioides (20 °C, pH 5.2, exposure time - 24 h, n = 3)

vice versa, glycosylation of protein molecule permits speaking of the controlled effect on such properties of biologically active molecules as their structure, stability and functions. Such modifications extend the areas of using glycoproteins in biochemical and biomedical technologies.

The significant role of carbohydrate component in the display of catalytic and kinetic properties of *C. cladosporioides* α-galactosidase was shown as a result of conducted investigations. It was first shown that the modification of α-galactosidase with sodium periodate was accompanied by a considerable decrease of the enzyme activity and stability. A comparative study of pH-, thermal stability, activity in respect of synthetic and natural substrates of the native and modified preparations gives ground for making a conclusion about the contribution of the carbohydrate component to the display of the structural and functional properties of the given glycosidase. We have also noted a decrease of not only enzyme activity, but also its stability in conditions of thermal denaturation. The native enzyme has displayed the highest activity in the acid range of pH, while neutral values were optimal for the modified enzyme. As a result of studying the chemical denaturation it was shown that the inhibiting effect of alcohols is intensified with the hydrocarbons chain lengthening. The results obtained will help to develop a strategy of the enzyme glycosylation for increasing its stability, which will allow us to use α-galactosidase of *C. cladosporioides* more efficiently in the processes of treatment of the soya and sugar-containing raw materials in various branches of food industry.

СТАБІЛЬНІСТЬ НАТИВНОЇ ТА МОДИФІКОВАНОЇ α-ГАЛАКТОЗИДАЗИ *Cladosporium cladosporioides*

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Модифікація вуглеводного компонента дозволяє встановити його роль у вияві структурно-функціональних властивостей глікопротеїнів. Представлена робота присвячена порівняльним дослідженням активності й стабільності нативної та модифікованої за допомогою окислення перйодатом натрію α-галактозидази *Cladosporium cladosporioides*. Для визначення активності α-галактозидази використовували синтетичний *n*-нітрофенільний субстрат, а також мелібіозу, рафінозу та стахіозу. Модифікація вуглеводного компонента спричи-

няла значний вплив на каталітичні властивості ензиму, відмічалося зниження як V_{max}, так і спорідненості ензиму щодо природних й синтетичних субстратів. Нативний ензим зберігає понад 50% від максимальної активності у діапазоні 20–60 °С, в той час як для модифікованого ензиму, за тих самих умов, цей діапазон звужується і становить 30–50 °С. Модифікована α-галактозидаза характеризується вищою термостабільністю в нейтральній зоні pH. Залишкова активність модифікованої α-галактозидази у разі обробки 70%-ми (v/v) метанолом, етанолом та пропанолом склала близько 30%. Близько 50% від вихідної активності реєструвалося за використання 40%-го етанолу і пропанолу, а також 50%-го метанолу. Вперше показано, що модифікація α-галактозидази *C. cladosporioides* перйодатом натрію супроводжувалася значним зниженням активності та стабільності ензиму, яке спричинене, можливо, конформаційними змінами в третинній та четвертинній структурі молекули протеїну.

Ключові слова: α-галактозидаза, *Cladosporium cladosporioides*, перйодат натрію, глікозилювання, модифікація.

СТАБИЛЬНОСТЬ НАТИВНОЙ И МОДИФИЦИРОВАННОЙ α-ГАЛАКТОЗИДАЗЫ *Cladosporium cladosporioides*

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Модификация углеводного компонента позволяет установить его роль в проявлении структурно-функциональных свойств гликопротеинов. Представленная работа посвящена сравнительным исследованиям активности и стабильности нативной и модифицированной с помощью окисления перйодатом натрия α-галактозидазы *Cladosporium cladosporioides*. Для определения активности α-галактозидазы использовали синтетический *n*-нитрофенильный субстрат, а также мелибиозу, раффинозу и стахиозу. Модификация углеводного компонента оказывала значительное влияние на каталитические свойства энзима, отмечалось снижение как V_{max}, так и сродства энзима по отношению к природным и синтетическим субстратам. Нативный энзим сохраняет более 50% от максимальной активности в диапазоне 20–60 °С, в то время как для модифицированного энзима в тех же условиях этот диапазон сужается и составляет 30–50 °С. Модифицированная α-галактозидаза характеризуется более высокой термостабильностью в нейтральной зоне pH. Остаточная активность модифицированной α-галактозидазы при обработке 70%-ми (v/v) метанолом, этанолом и пропанолом составила около 30%. Около 50% от исходной активности регистрировалось при использовании 40%-го этанола и пропанола, а также 50%-го метанола. Впервые показано, что модификация α-галактозидазы *C. cladosporioides* перйодатом натрия сопровождалась значительным снижением активности и стабильности энзима, вызванным, возможно, конформационными изменениями в третичной и четвертичной структуре молекулы протеина.

Ключевые слова: α-галактозидаза, *Cladosporium cladosporioides*, перйодат натрия, гликозилирование, модификация.

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