ЕКСПЕРИМЕНТАЛЬНІ РОБОТИ

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THERMAL STABILITY OF *Cryptococcus albidus* α-L-RHAMNOSIDASE

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Yeast as well as micromycetes α-L-rhamnosidases, currently, are the most promising group of enzymes. Improving of the thermal stability of the enzyme preparation are especially important studies. Increase in stability and efficiency of substrate hydrolysis by α-L-rhamnosidase will improve the production technology of juices and wines. The aim of our study was to investigate the rate of naringin hydrolysis by α-L-rhamnosidase from Cryptococcus albidus, and also some aspects of the thermal denaturation and stabilization of this enzyme. We investigated two forms of α-L-rhamnosidase from C. albidus, which were obtained by cultivation of the producer on two carbon sources – naringin and rhamnose. A comparative study of properties and the process of thermal inactivation of α-L-rhamnosidases showed that the inducer of synthesis had no effect on the efficiency of naringin hydrolysis by the enzyme, but modified thermal stability of the protein molecule. Hydrophobic interactions and the cysteine residues are involved in maintaining of active conformation of the α-L-rhamnosidase molecule. Yeast α-L-rhamnosidase is also stabilized by 0.5% bovine serum albumin and 0.25% glutaraldehyde.

K e y w o r d s: Cryptococcus albidus, α-L-rhamnosidase, thermal inactivation, thermal stability, naringin, rhamnose.

α-L-Rhamnosidase which is capable to cleave non-reduced terminal L-rhamnose residues presented in both natural and synthetic glycosides, oligosaccharides, polysaccharides, glycolipids is one of the important enzymes used in juice- and wine-making [1]. The flavonoids derivatives such as rutin, neohesperidin, naringin, quercitrin, hesperidin as well as ginsenosides and asiaticoside may serve as natural substrates for this enzyme. These properties of α-L-rhamnosidase allow its use in various industries. The enzyme hydrolyzes terpene glycosides such as rutinoside and thus contributes to the release of aromatic compounds enhancing the flavor of juice and wine. Cleavage of bioflavonoid naringin allows removing the bitterness of citrus juices, especially in grapefruit juice [2]. α-L-Rhamnosidase is widely used in the chemical industry to obtain rhamnose and natural glycosides [3]. It is significant that the enzymes involved in all these processes are stable at high temperatures in concentrated solutions of substrates and products of reactions.

From the point of view of production, yeast is the best producer as it is characterized by a high growth rate, resistance to outside microflora, ability to assimilate a wide range of food sources and does not pollute the air in contrast to micromycetes. Thus far, α-L-rhamnosidase activity was found in *Saccharomyces cerevisiae* Tokaj 7, *Hansenula anomala*, *Debaryomyces ploymorphus* [4], *Aureobasidium pullulans*, *Candida guilliermondii* and *Pichia angusta* X349 [5]. However, all these producers synthesize intracellular α-L-rhamnosidases, that complicates work with them.

Previously [6] the promising strain of *Cryptococcus albidus* 1001 as a producer of an extracellular α-L-rhamnosidase was selected by screening of yeast museum cultures of the Department of Physiology of industrial microorganisms IMV NASU. The purified enzyme preparation was obtained from *C. albidus* culture supernatants [7].

The studies of the physicochemical properties of α-L-rhamnosidase from *C. albidus* have shown

that the enzyme exhibits high thermal stability in a wide temperature range of 20 °C up to 70 °C at pH 4.0-6.0 [7]. The study of the enzyme preparation composition [8] showed that aspartic, glutamic and glycine are dominant amino acids and 1.2% of cysteine and up to 31% of hydrophobic amino acids are also present. The hydrophobic amino acids and carbohydrates (5%) may attribute to the high stability of the enzyme *C. albidus.* Since this enzyme has a high affinity for naringin (K_m 0.77 mM), it opens up prospects for its use in juice and wine production.

The aim of this work was to carry out a comparative study of thermal stability of the preparations of *C. albidus* α-L-rhamnosidase obtained by the yeast growing on different carbon sources and to investigate the mechanism of their thermal inactivation.

Materials and Methods

Two preparations of extracellular *C. albidus* α-L-rhamnosidase, such as α-L-Rham R and α-L-Rham N obtained by growing the producer respectively on media with rhamnose (5 g/l) or naringin (5 g/l) as the sole carbon sources, were used in this study. These preparations were characterized by different purification degree: 1) supernatant of culture fluid (CF) 0.3 U/mg of protein, 2) partially purified by gel filtration on Toyoperl TSK HW-60 enzyme preparation (PP) 5.0 U/mg of protein (purification degree 16.6) and 3) enzyme preparation additionally purified by ion exchange chromatography on DEAE-Toyopearl 650-s (Toyo Soda, Japan) according to the previously developed procedure 12.5 U/mg of protein (purification degree 42) [7].

α-L-Rhamnosidase activity was determined by the method of Davis [9] using naringin as substrate. Protein was determined by Lowry assay [10].

Thermal inactivation of α-L-rhamnosidase was studied at 60 and 65 °C, pH 5.2 (0.1 M phosphate-citrate buffer (PCB)). The study of thermal inactivation kinetics included the following steps: the enzyme samples (3 U/ml) in 3 ml of the appropriate buffer were kept at given temperature for 1.5-3 h; the aliquots in 0.1 ml were collected in definite intervals (10-30 min) for measurement of residual α-L-rhamnosidase activity. Inactivation rate constant was determined from the slope of the line on the plot showing dependence of the natural logarithm of the residual enzyme activity value on time.

Enzyme molecular weight (Mw) was determined by gel filtration on Sepharose 6B column (1.3×52 cm) equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution of protein was monitored by absorbance at 280 nm and enzyme activity. Molecular weight was determined using markers such as bovine serum albumin (BSA) (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.0 kDa; Pharmacia, Sweden).

Enzyme treatment with glutaraldehyde was carried out as follows: 10 µl of 25% glutaraldehyde solution was added to 1 ml of the purified enzyme solution (8 U/ml) and the mixture was incubated at room temperature for 60 min. The remaining reagent was removed by gel filtration on Sepharose 6B. Thermal inactivation was carried out as described above.

The effect of ionic strength on α-L-rhamnosidase thermal stability was studied as described above using phosphate-citrate buffer at concentration range from 0.01 to 1.0 M, pH 5.2. The effect of enzyme purification degree on its thermal stability was studied at 0.5-5 U/mg of protein. The effect of substrate concentration on the activity and the thermal stability of α-L-rhamnosidases were evaluated under standard conditions with 0.1M PCB, pH 5.2 and naringin concentrations range from 0.25 to 4.0 mM. BSA at concentration 0.5% was used to stabilize enzyme preparations.

Reagents of thiol-disulfide exchange such as dithiothreitol, mercaptoethanol, glutathione at concentration 10^{-3} M were used in the thermal inactivation experiments. Ellman's reagent was used to determine the number of sulphydryl groups [11].

All experiments were performed in 3-5 replicates. Statistical analysis of the experimental results was carried out using Microsoft Excel 7.0 and the Student's *t*-test at 5% of statistical level [12].

Results and Discussion

Recently the mechanism of biocatalysts denaturation has been studied widely. The results of these studies allow us to predict the behavior of the enzyme in the changing conditions of the reaction medium and to develop a strategy to increase the stability of enzyme preparations used in various biotechnological processes. The presence of glycosylated sites in the polypeptide may determine the stability of the enzyme at high temperature and other factors of aggressive environment and may lead to formation of supramolecular structures. Since enzyme glycosylation may directly depend on the presence of carbohydrates in the media, the use of

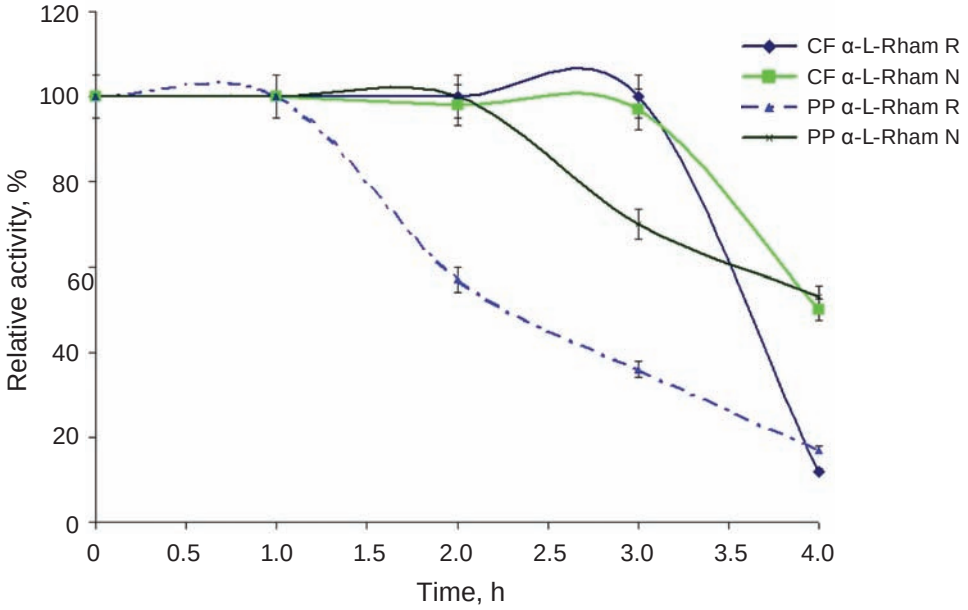

Fig. 1. Kinetic curves of thermal inactivation of C. albidus α-L-rhamnosidase with varying degree of purification at 65 °C (CF - the supernatant of culture fluid, PP - partially purified enzyme preparation, obtained by gel filtration on Toyoperl TSK HW-60), n = 3

various sources of carbohydrate supply during biosynthesis of inducible enzymes allows obtaining isoforms which differ in degree of glycosylation and stability. α-L-Rhamnosidases of *C. albidus* obtained by growing on two carbon sources such as naringin and rhamnose and differing in degree of purification were used in our experiments.

Study of the process of thermal inactivation of *C. albidus* α-L-rhamnosidase of varying degrees of purification showed that it depended on the carbon source which was used for culturing the producer. Thus, α-L-Rham N was more stable during the first 3 hours of incubation and then the activity of both the supernatant and partially purified preparation was reduced to 50% (Fig. 1). α-L-Rham R exhibits a lower stability. Although activity of the supernatant was not altered during the first 3 hours of incubation, it dropped rapidly to 90% of the initial value during the next hour. The activity of the purified enzyme preparations began to decrease after one (α-L-Rham R) or two (α-L-Rham N) hours of incubation. The obtained results may depend on protein glycosylation conditions such as varying carbon sources in the growth medium and protective effect of the impurities of the crude preparations. The experiments with purified preparations also showed that the introduction of 0.5% neutral protein such as BSA to the reaction mixture stabilized α-L-Rham N and α-L-Rham R in thermal denaturation conditions increasing the enzyme half-life by 50% (Fig. 2).

All further experiments were performed using purified preparations of α-L-Rham N and α-L-Rham R. It was shown during study of naringin hydrolysis that α-L-Rham N hydrolyzed high substrate concentrations more efficiently (optimum activity was observed at 3.5 mM) (Fig. 3).

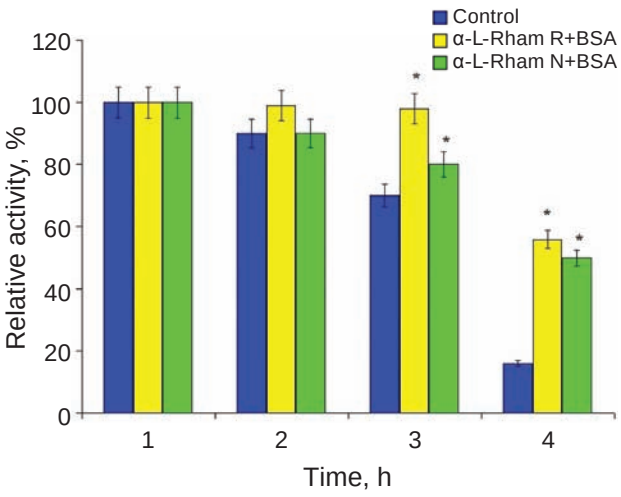

*Fig. 2. Dependence of C. albidus α-L-Rham R and α-L-Rham N activity on time at 65 °C in the presence of 0.5% BSA. * $P \leq 0.05$, n = 3*

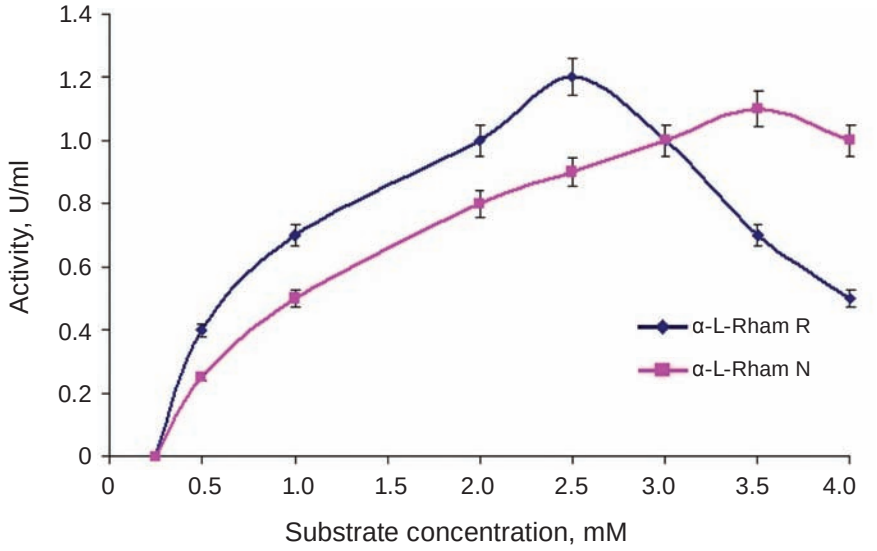

Fig. 3. Dependence of enzyme activity on the naringin concentration, pH 5.2, 37°C, n = 5

Studies of the time course of naringin hydrolysis at various temperatures (Fig. 4) showed that *C. albidus* α-L-Rham R completely hydrolyzed 2 mM naringin during 3 h at 60 °C. The time of hydrolysis increased to 4 hours when the temperature lowered to 50 °C. A similar effect was observed for α-L-Rham N. The obtained results indicate a high potential of the use of these preparations in biotechnological processes. It was also shown that thermal inactivation of α-L-Rham R and α-L-Rham N was accelerated in the presence of 0.5% rhamnose (Fig. 5). Thus, it should be noted that the enzyme stability is decreased with increasing concentrations of rhamnose – the resulting product of naringin hydrolysis, thus a well-timed removal of rhamnose from the reaction media is required.

The study of kinetics of *C. albidus* thermal inactivation at 60 °C (Fig. 6) showed a high stability of both enzyme forms under these conditions, although the α-L-Rham N exhibits a higher initial activity as was indicated above. However, the time courses of thermal denaturation of purified preparations have common characteristics. An analysis of thermal inactivation curves at 20-60 °C allows us to suggest that one or more "stable and unstable" forms of this enzyme should be considered. Thus,

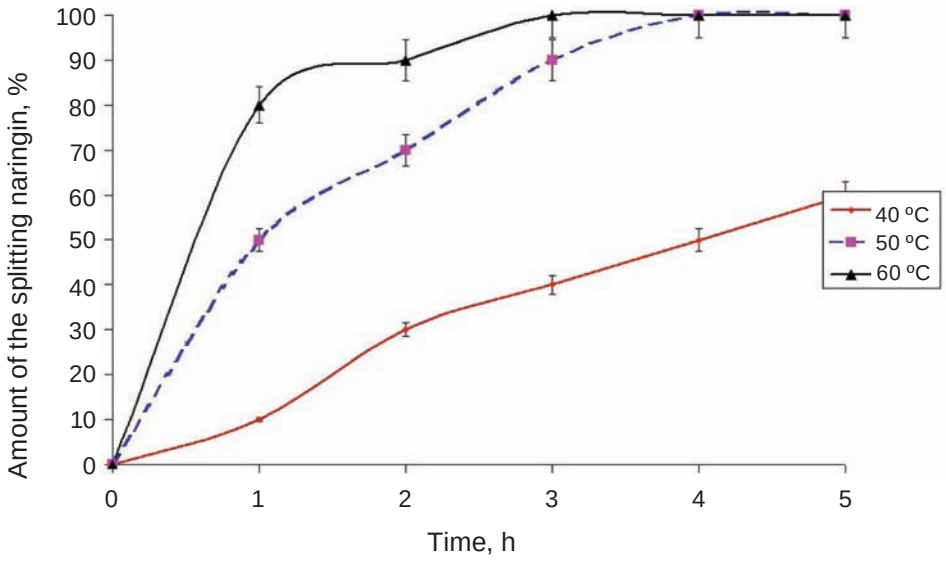

Fig. 4. Time course of naringin hydrolysis by α-L-Rham R C. albidus at different temperature, n = 5

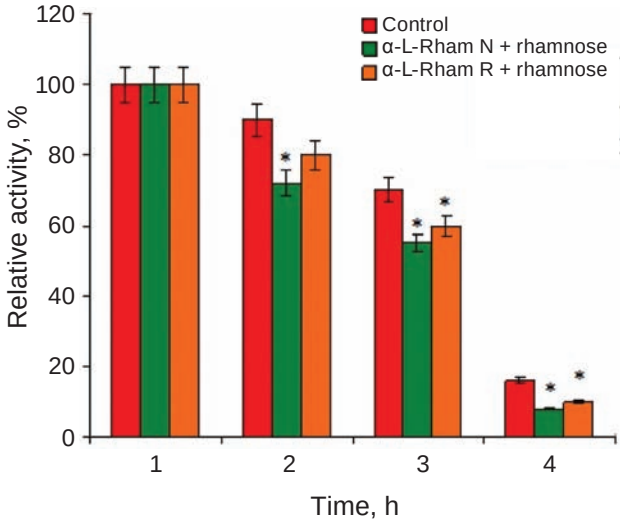

*Fig. 5. Time course of α-L-Rham R and α-L-Rham N C. albidus thermal inactivation in the presence of rhamnose 0.5% (pH 5.2, 65°C). * $P \leq 0.05$, n = 3*

the inactivation results from a series of successive conversions with formation of active intermediate structure. Irreversible first order inactivation of the successive stages was observed at temperature of 70-80 °C. The thermal inactivation kinetic constants for α-L-Rham R and α-L-Rham N were calculated and found to be 1.3×10^{-6} and 1.6×10^{-6} c^{-1} at 60 °C, 5.1×10^{-5} and $6,4\times10^{-5}$ c^{-1} at 65 °C, 8.7×10^{-5} and 1.4×10^{-4} c^{-1} at 70 °C, respectively.

A decrease of Mw of α-L-Rham R and α-L-Rham N (from 50 to 30 kDa) was observed during denaturation. It may be assumed that the enzyme active form exists in the associated state, however these protein aggregates are broken-down under the temperature effect, which contributes to the activity loss.

An analysis of the time course of thermal inactivation of both forms of *C. albidus* α-L-rhamnosidase in solutions with varying ionic strength showed (Fig. 7) that hydrophobic interactions play a dominant role in maintaining the active protein molecule conformation. That was evidenced by a slight decrease in enzyme stability as the solution concentration was increasing. These results allow one to select reagents and conditions for *C. albidus* α-L-rhamnosidase stabilization.

Resistance of enzymes to thermal denaturation can be enhanced by the introduction of intra- and intermolecular cross-links. Biocatalysts based on cross-linked aggregates exhibit enhanced stability and better stereochemical accessibility for immobilizing agents [1]. They are characterized by mechanical, chemical and thermal stability, high activity and usually do not require the insertion of foreign non-enzymatic substances. Glutaraldehyde is the most commonly used cross-linking agent owing to its ability to form polymers acting as crosslinkers with varying length of bridges. It was shown that

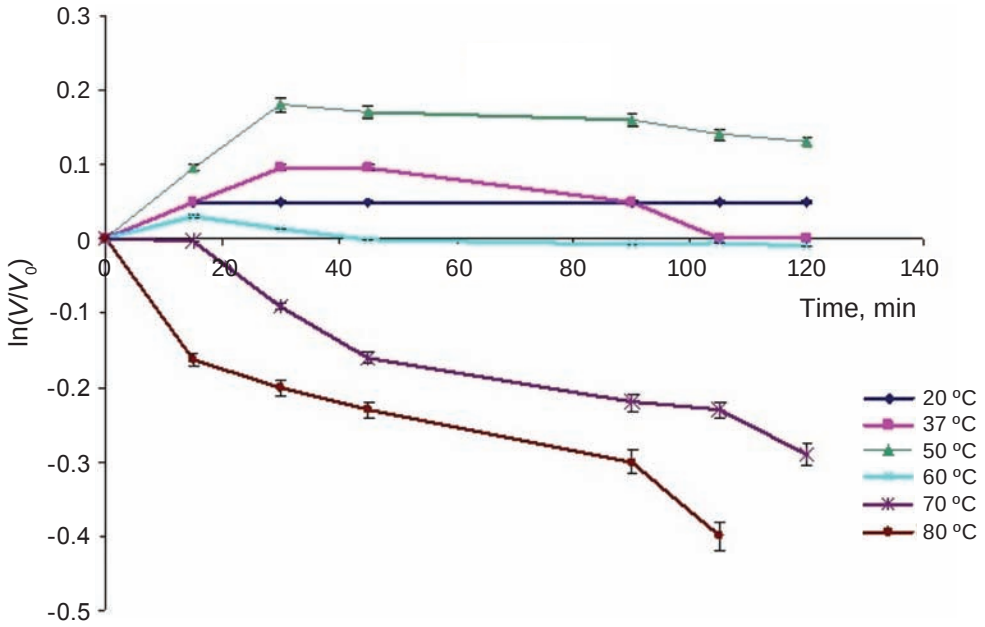

Fig. 6. Kinetics of thermal inactivation of C. albidus α-L-Rham N at different temperature, n = 5

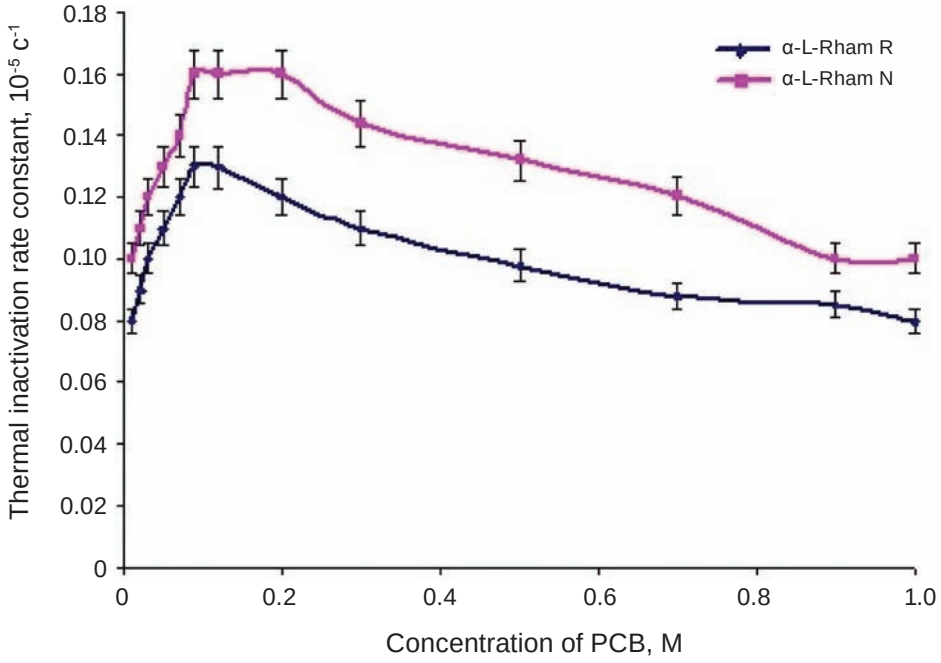

Fig. 7. Dependence of α-L-rhamnosidases C. albidus thermal inactivation rate constant on concentration of PCB (pH 5.2, 60 °C), n = 3

the enzyme conformation structure which exhibits high stability at 65 °C was formed at concentration of glutaraldehyde of 0.25% (Fig. 8).

The formation of a rigid structure of the enzyme active site may not be crucial for the enzyme activity or possible aggregation of molecules preventing thermal denaturation. Obtained results may serve as a base for creating stable immobilized form of *C. albidus* α-L-rhamnosidase.

Various interactions and the presence of amino acid residues may contribute to the protein molecule stability. Thus sulfhydryl groups of cysteine residues are very important for maintaining the active protein conformation. These groups are effective nucleophilic agents with high reactivity. The quantity of SH-groups in the molecule of *C. albidus* α-L-rhamnosidase was calculated and was found to be 8.64×10^{-7} mM per mg of protein. The study of thermal denaturation process in the presence of mercaptoethanol, dithiothreitol and glutathione was carried out for clarification of the role of thiol-disulfide exchange components in the stability of *C. albidus* α-L-rhamnosidase. It was shown (Fig. 9) that the presence of thiol-disulfide exchange agents accelerated significantly the process of inactivation of both *C. albidus* α-L-rhamnosidase forms, but α-L-Rham N mostly. This data indirectly indicates conformational differences between the enzymes

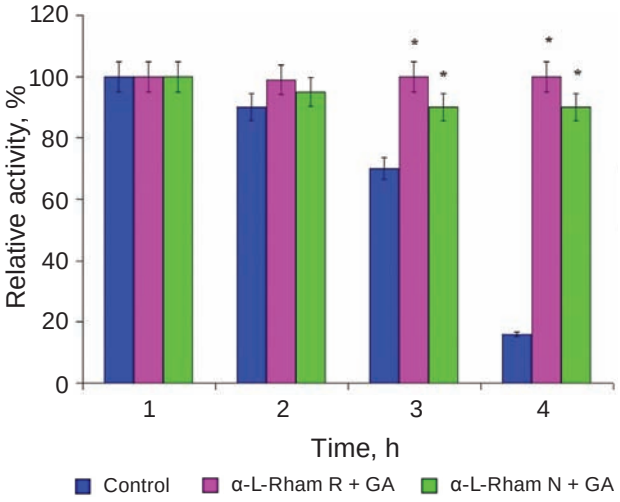

*Fig. 8. Thermal stability of C. albidus α-L-rhamnosidases in the presence of glutaraldehyde 0.25% (pH 5.2, 65 °C). * P ≤ 0.05, n = 5*

(accessibility of cysteine or other amino acid residues) obtained under different conditions of culture producer growth.

Thus, it was found that the thermal stability of *C. albidus* α-L-rhamnosidases is directly dependent on both the degree of enzyme purification and inducer of synthesis. Although it should be noted that α-L-Rham N exhibits a higher potential. Hydrophobic interactions and the presence of cysteine residues

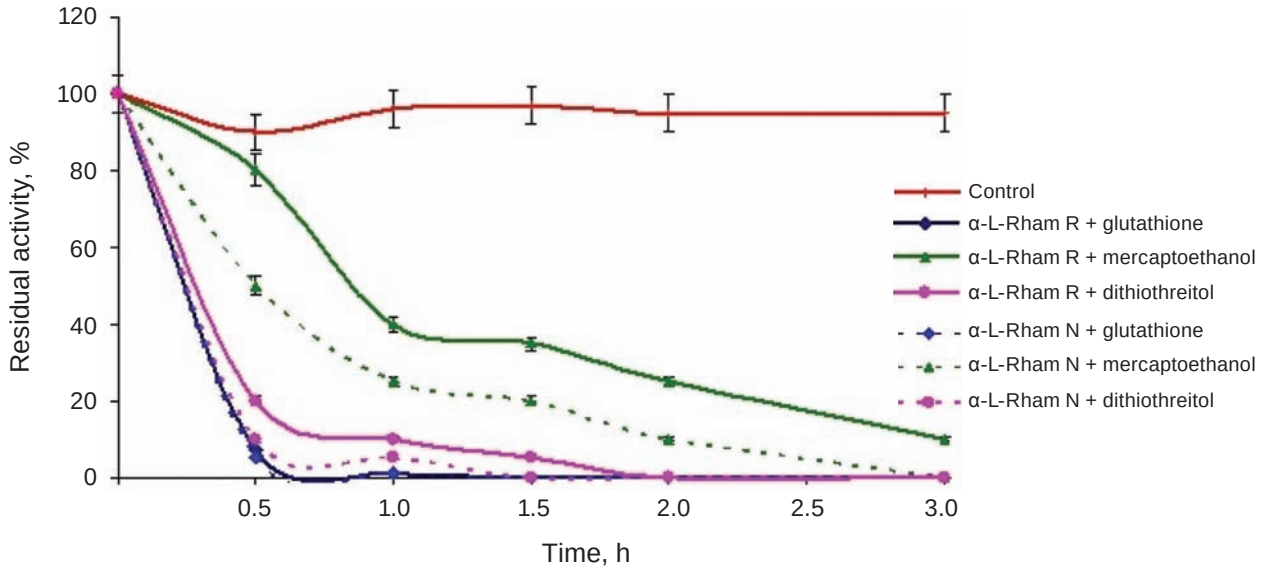

Fig. 9. Dependence of C. albidus α-L-rhamnosidases activity on time under the presence of thiol-disulfide exchange agents (10^{-3} M) at 60 °C, pH 5.2, n = 3

made a significant contribution to the structure stabilization of both enzyme forms. It was shown that *C. albidus* α-L-rhamnosidase hydrolyzes naringin effectively at high concentrations and increase temperatures. Neutral protein (0.5%) and glutaraldehyde (0.25%) stabilize the yeast α-L-rhamnosidase regardless of production method. The obtained results allow us to recommend this enzyme for use in biotechnological processes.

ТЕРМОСТАБІЛЬНІСТЬ α-L-РАМНОЗИДАЗИ *Cryptococcus albidus*

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α-L-Рамнозидази як дріжджів, так і мікроміцетів є на сьогодні найперспективнішою групою ензимів. Особливо важливими є дослідження, спрямовані на підвищення термостабільності ензимних препаратів. Вивчення шляхів підвищення термостабільності та ефективності гідролізу субстрату α-L-рамнозидазою дозволить удосконалити технологію виробництва соків та вин. Метою нашої роботи було дослідити швидкість гідролізу нарингіну α-L-рамнозидазою *Cryptococcus albidus*, а також деякі аспекти процесу термоденатурації та стабілізації ензиму. Було досліджено 2 форми α-L-рамнозидази *C. albidus*, одержані за вирощування продуцента на різних джерелах вуглецю – нарингіні та рамнозі. Порівняльне вивчення властивостей та процесу термоінактивації цих α-L-рамнозидаз показало, що індуктор синтезу не впливає на ефективність гідролізу нарингіну ензимом, однак вносить зміни в термостабільність протеїнової молекули. У підтриманні активної конформації молекули α-L-рамнозидази беруть участь гідрофобні взаємодії та залишки цистеїну. Також можна стабілізувати дріжджову α-L-рамнозидазу *C. albidus* 0,5%-им бичачим сироватковим альбуміном і 0,25%-им глутаровим альдегідом.

Ключові слова: *Cryptococcus albidus*, α-L-рамнозидаза, термоінактивація, термостабільність, нарингін, рамноза.

ТЕРМОСТАБИЛЬНОСТЬ α-L-РАМНОЗИДАЗЫ *Cryptococcus albidus*

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α-L-Рамнозидазы как дрожжей, так и микромицетов являются в настоящее время одной из перспективных групп энзимов. Особенно

важны исследования, направленные на повышение термостабильности энзимных препаратов и эффективности гидролиза ими субстрата, что позволит усовершенствовать технологию производства соков и вин. Целью работы было изучить скорость гидролиза нарингина α-L-рамнозидазой *Cryptococcus albidus*, а также некоторые аспекты процесса термоденатурации и стабилизации энзима. Были исследованы две формы α-L-рамнозидазы *C. albidus*, полученные при выращивании продуцента на различных источниках углерода – нарингине и рамнозе. Сравнительное изучение свойств и процесса термоинактивации этих α-L-рамнозидаз показало, что индуктор синтеза не влияет на эффективность гидролиза нарингина энзимом, однако изменяет термостабильность протеиновой молекулы. В поддержании активной конформации молекулы α-L-рамнозидазы участвуют гидрофобные взаимодействия и остатки цистеина. α-L-Рамнозидазу *C. albidus* также можно стабилизировать 0,5%-ым бычьим сывороточным альбумином и 0,25%-ым глутаровым альдегидом.

Ключевые слова: *Cryptococcus albidus*, α-L-рамнозидаза, термоинактивация, термостабильность, нарингин, рамноза.

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