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THERMOSTABILIZATION OF *Eupenicillium erubescens* AND *Cryptococcus albidus* α-L-RHAMNOSIDASES BY CHEMICAL REAGENTS

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The aim of the research was a comparative study of the thermal stability of native and modified by various methods α-L rhamnosidases of *Eupenicillium erubescens* and *Cryptococcus albidus* for improving the stability of enzymes. Denaturation of native and modified enzymes were performed at 65 °C, pH 5.2. Enzyme activity was determined using *p*-nitrophenyl-α-L-rhamnopyranoside and naringin. It is found that in the treatment by polyethylenglycol PEG 1500, dextrans 500 and 70 T thermostability of α-L-rhamnosidases tested decreases, while modification with polyethylenglycol 20000 leads to increase thermal stability of the *E. erubescens* enzyme to 280% and C. *albidus* to 150%. Comparative study of the thermal stability of the native and modified by cellulose and its derivatives α-L-rhamnosidases of *C. albidus* and *E. erubescens* showed that at concentrations cellulose of 5–15 μg / 10 μg protein protective effect of polymers on enzymes was investigated was observed. Hydrophobic modifications using succinic anhydride also can slow down the denaturation of α-L-rhamnosidases tested under experimental conditions. These stabilized *C. albidus* and *E. erubescens* α-L- rhamnosidases can be used in biotechnological processes.

Key words: α-L-rhamnosidase, *Eupenicillium erubescens, Cryptococcus albidus*, thermal stabilization of enzymes.

One of the important physicochemical protein properties is their resistance to denaturing impact of different nature. Data on the enzymes stability are needed for practical work with them and for usage in biotechnological industries. Enzymes folding-defolding research allows important information obtaining about their stability and activity.

α-L-Rhamnosidase (EC 3.2.1.40) is an enzyme that hydrolytically cleaves non-reduced terminal L-rhamnose residues present in synthetic and natural glycosides, oligo-, polysaccharides, glycolipids and various glycoconjugates. The use of this enzyme in various industry branches, including food industry — to remove the bitterness in citrus juices and improve the wine quality, pharmaceutical industry — for new drugs obtaining, and chemical industry — to obtain rhamnose [1], requires the thermostable α-L-rhamnosidases involvement in these productions.

The problem of these biocatalysts stabilization should be solved firstly for the enzymes successful use. Most enzymes require the presence of some non-peptide substances (cofactors) for catalytic activity appearance. Among the latter there are two groups: metal ions (and some inorganic anions) and coenzymes that are organic substances. Previously we have studied the impact of series of compounds on the α-L-rhamnosidases of *C. albidus* and *E. erubescens* activity and stability [2, 3, 4]. It is found that some carbohydrates and substrates of these enzymes can significantly extend their “half-lifetime” period.

As mechanisms of α-L-rhamnosidases stabilization is almost unexplored today, the purpose of the work was to study different approaches (stabilization by hydrophilic polymers, immobilization on cellulose and its derivatives, hydrophobic and group-specific modification) to obtain stabilized α-L-rhamnosidases of *C. albidus* and *E. erubescens*.

Materials and Methods

Extracellular α-L-rhamnosidases of *Cryptococcus albidus* 1001 and *Eupenicillium erubescens* 248 obtained due to clearing in charged and neutral TSK-gels as described previously were used in the work [5, 6].

To determine the α-L-rhamnosidase activity, 0.2 ml of 0.1 M phosphate-citrate buffer (PCB), pH 5.2, and 0.1 ml of 0.01 M solution of the substrate in PCB were added to 0.1 ml of enzyme. The reaction mixture was incubated for 10 min at 37 °C. The reaction was stopped by adding of 2 ml of 1 M sodium bicarbonate. To the retention sample the same components were added, but in reverse order. The amount of relesed *p*-nitrophenol as a resalt of hydrolysis was determined colorimetrically by the adsorption at 400 nm [7]. One unit of enzyme activity was defined as the amount of the enzyme that releases 1 μmol of *p*-nitrophenol per 1 min under experimental conditions. α-L-Rhamnosidase activity was determined using naringin by Davis method [8].

α-L-rhamnosidase thermal inactivation was examined at 65 °C, pH 5.2 (0.1 M PCB). The samples of native and modified enzyme (1.5 U/ml) in 1 mM PCB, pH 5.2, were kept at a given temperature for 3 hours. At certain intervals (15–180 min) aliquot of 0.1 ml were taken and α-L-rhamnosidase activity was measured.

To stabilize the α-L-rhamnosidase, dextrans T500 and T70, polyethyleneglycols (PEG) 1500, PEG 4000 0, PEG 6000, PEG 20000 (5 g/l), cetyltrimethylammonium bromide (CTAB) at concentrations of 10^{-2}–10^{-6} M, *p*-chloromercuribenzoate (*p*-CMB) (10^{-3}–10^{-5} M), glycine (0.1 M), 2,6 diaminopimelic acid (10^{-3} M), sodium diethylpyrocarbonate (DEPC) (1 %), succinic anhydride (10^{-2}–10^{-5} M), dithiothreitol (10^{-3} M), L-cysteine (10^{-3} M) were used.

The treatment with dextrans and PEG was performed as follows: 1 ml of enzyme solution (1 mg/ml) in PCB, pH 5.2, was added to 1 ml of polymer solution (50 mg/ml) in PCB, pH 5.2. The reaction was performed at 20 °C for 24 hours with constant stirring. Then enzyme polymer complexes were separated from low molecular weight reagents by gel filtration on Sepharose 6B [9].

The reaction with CTAB was carried out in PCB solution, pH 6.0. 1 ml of CTAB at concentration of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} M was added to 1 ml of enzyme (1 mg/ml). After incubation for 24 hours the remaining reagent was separated by dialysis against PCB [10].

Immobilization of enzymes on cellulose, microcrystalline cellulose (MC-cellulose), carboxymethyl cellulose (CM-cellulose) diethylaminoethyl cellulose (DEAE-cellulose) and Servacel CM 32 was carried out by mixing polymers with enzymes in PCB at a final concentration of 5, 10, 15, 20 and 25 mg per 10 mg of protein. The mixture was kept at 32 °C for 24 hours with constant stirring, insoluble component was separated by centrifugation (10 000 g, 5 min).

Succinic anhydride-assisted hydrophobic modification was performed as followed: to the incubation mixture with 1 mg of protein, succinic anhydride of various concentrations (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} M) was added. Sampls were kept for 8 hours with constant stirring. The modified enzyme was separated from low molecular weight compounds by dialysis against PCB, pH 5.2 [11].

The incubation of enzymes (1 mg/ml) with *p*-CMB, glycine, 2,6-diaminopimelic acid, DEPC, dithiothreitol, L-cysteine at appropriate concentrations was carried out in PCB (pH 5.2) at 20 °C for 24 h.

The activity of obtained enzymes was studied at 37 °C and under thermo-denaturation.

To determine the number of sulfhydryl groups Elman reagent was used [12].

All experiments were performed in 5–7 repetitions. Statistical analysis was performed by methods of variation and correlation statistics using Student t-test [13]. Average values and standard errors ($M \pm m$) were calculated. The values at $P < 0.05$ were regarded as reliable. The results presented graphically were processed using Microsoft Excel 2003.

Results and Discussion

Stability of industrially important proteins increasing which enables biocatalysts efficient use for a long time with minimal loss of activity is the priority of modern applied enzymology. To solve the problem of enzymes denaturation in unnatural conditions of the reaction medium, various approaches can be applied, including chemical modification which has a significant impact on the hydrophilic-lipophilic balance of protein surface.

A number of studies [9, 11, 14] demonstrated that changing of protein hydrophilic-lipophilic balance by chemical

modification leads to changes in enzyme activity and stability in water and organic media. However, until now, majority of essential aspects of protein denaturation mechanism have not been studied enough, that makes it impossible to offer universal ways of their stabilization.

Hydrophobic modification can lead both to increase and to reduce the thermal stability of proteins. For example, if horseradish peroxidase amino groups were modified by anhydrides of acetic, propionic, valeric and maleic acids, an increase in thermal stability of modified enzymes compared to the native has been observed in all cases [9, 14], while for other enzymes the protein hydrophilization is expedient [11]. Currently, there are no data concerning the study of modified α-L-rhamnosidases. Therefore, a comparative study of native and modified α-L-rhamnosidases of *C. albidus* and *E. erubescens* thermal stability has been carried out.

α-L-Rhamnosidases treatment with polymers has shown (Fig. 1, *A, B*) that in the case of dextrans 70 T and 500 T use, the acceleration of both producers' enzymes thermodenaturation was observed.

Today, one of the common modifiers of peptide structure compounds is PEG, [15] it is registered by FDA (Food and drug administration of USA) as a substance for the manufacture of medicinal and cosmetological preparations and can be the component of food. PEG-assisted chemical modification of proteins does not lead to significant changes in their tertiary and quaternary structure, but it causes the physical and chemical transformation which is reached by alkylation of protein molecule. One of the most important parameters of PEG modified molecules is their hydrophilicity, which forms a fundamentally new physical and chemical properties of the modified peptide. Hydrophilic hydration of modified peptide occurs through hydrogen bonds that are formed between water molecules and oxyethylic groups of PEG. This hydration promotes "water cloud" formation around the PEG + protein modified molecule, as a result of its hydrodynamic radius significantly increases. This hydration shell, on the one hand, significantly increases the preparation solubility and availability, and on the other hand protects the molecule against other proteins (neutralizing antibodies, complement). Longer PEG chains cause greater duration of the half-lifetime of PEG-peptide conjugate, and its stability. Another important factor is branched PEG molecule structure, providing a more stable protein modification. Stabilizing effect of modifier is defined also by the degree of its polymerization. Thus, the greatest effect was observed when α-L-rhamnosidase of *E. erubescens* was treated with PEG 20 000: thermal stability was increased by 280% for 180 min of incubation. PEG 4 000 and PEG 6 000 effect was slightly lower, the activity, if treated with them, was at the level of 100–120 %, while the modification by PEG 1 500, by contrast, accelerated the enzyme thermodenaturation compared to control.

Similar results were observed for *C. albidus* α-L-rhamnosidase (Fig. 1, *B*). The treatment with PEG 20 000 was especially effective, which resulted in increased by 150% thermal stability (180 min of incubation). PEG 4 000 and PEG 6 000 modified α-L-rhamnosidase of *C. albidus* retained 90–100% activity during this time, while enzymes treated with 70 T and 500 T dextrans lost their activity within

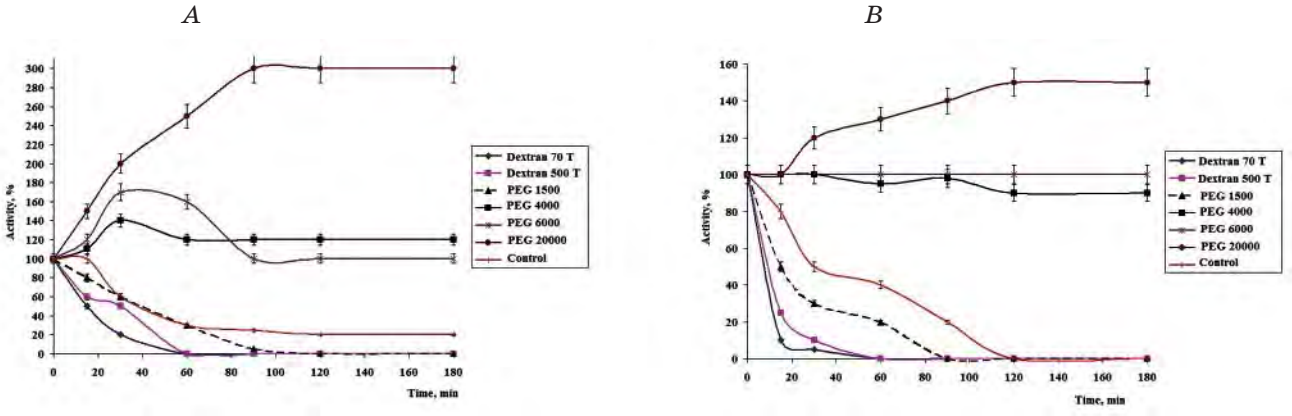

Fig. 1. **Dependence of *E. erubescens* (*A*) and *C. albidus* (*B*) α-L-rhamnosidase activity on time in polymers presence (pH 5.2, t = 65 °C)**

60 min of incubation, and treated with PEG 1 500 — within 90 min.

Thus, hydrophilic nonionic PEG-assisted modification accompanied by hydration shell on the protein surface formation is reasonable in the performance of a task of enzyme stabilization in aqueous solutions and can be used in the future to produce modified forms of α-L-rhamnosidases. However, these forms of the enzyme obtaining and PEG effect study on the degree of protein molecule hydration and its activity expression need further research.

Also the possibility of enzymes stabilization using cationic surfactants (CTAB) has been studied. It is known that CTAB can cause conformational changes in protein secondary structure [10]. Formation of protein-CTAB complex leads to a decrease the portion of α-helical chains, and this, in turn, promotes thermal stability increasing. At low surfactant concentrations the interaction is of electrostatic nature due to surfactant ions binding by oppositely charged protein groups, and in high concentrations the central role is played by hydrophobic interactions. It should be noted that the caused by CTAB enzymes hydrophobicity was accompanied by a decrease in their activity (Fig. 2), and formed complexes were more thermally labile than the native enzymes and completely inactivated at 65 °C for 1 hour.

Hydrophobic modification of investigated enzymes by other reagents was more successful. α-L-Rhamnosidases of *C. albidus* and *E. erubescens* inactivation period has increased due to succinic anhydride acylation of lysine amino groups. This modification stabilizes the enzymes active form by subunit interactions inhancing. The maximum effect was observed when α-L-rhamnosidases of *E. erubescens* and *C. albidus* were treated with succinic anhydride at concentration of 10^{-2} M (Fig. 3). The activity was about twice as much as activity of untreated enzymes. In addition, the little stabilizing effect was found for α-L-rhamnosidase of *E. erubescens* at succinic anhydride concentration of 10^{-3} M. Similar results were obtained also for α-galactosidases of *A. niger*, *C. cladosporioides* and *P. canescens* [11].

Enzymes treatment with 2,6-diaminopimelic acid, that is lysine precursor, aids *E. erubescens* α-L-rhamnosidase stabilization (Fig. 4, *A*), that could be seen during the first 90 min of incubation, whereas for *C. albidus* α-L-rhamnosidase protective effect was observed after 90 min of incubation. 2,6-Diaminopimelic acid protective effect can be explained probably by amino acids binding with surface residues, which in turn provided the intermolecular crosslinking and helped to preserve the protein molecule active conformation.

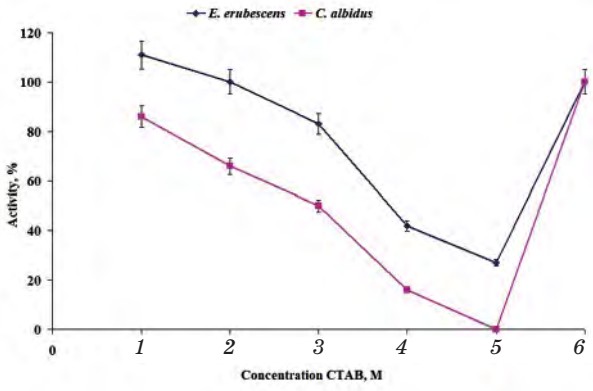

Fig. 2. **Dependence of *E. erubescens* and *C. albidus* α-L-rhamnosidase activity on CTAB concentration (t = 20 °C, 30 min):**
1 — 10^{-6}; *2* — 10^{-5}; *3* —10^{-4}; *4* — 10^{-3}; *5* — 10^{-2}; *6* — control

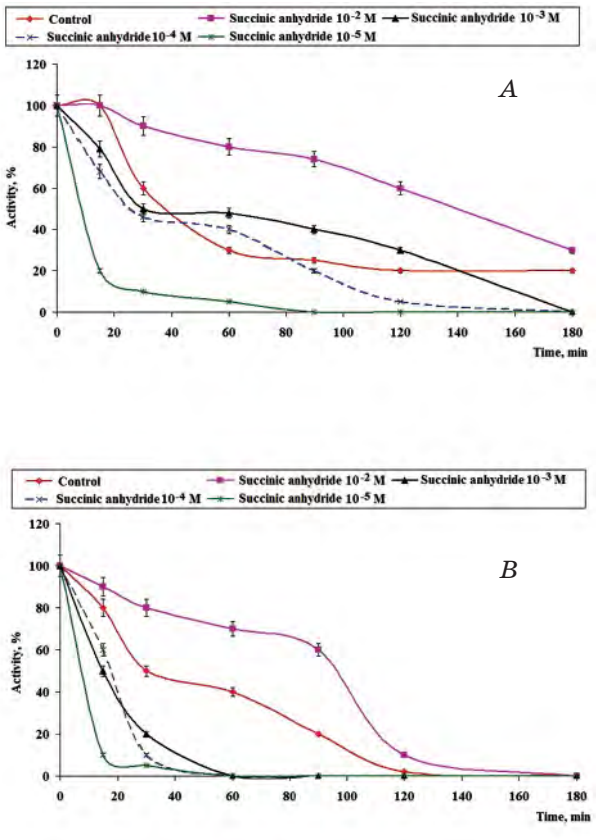

Fig. 3. **Dependence of *E. erubescens* (*A*) and *C. albidus* (*B*) modified by succinic anhydride α-L-rhamnosidase activity on the time of thermal inactivation (pH 5.2; t = 65 °C)**

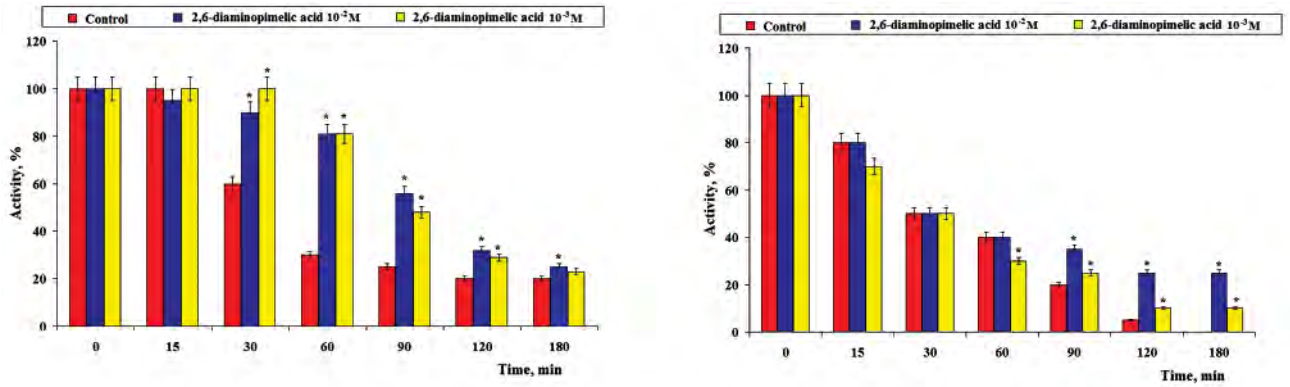

Fig. 4. **Dependence of *E. erubescens* (*A*) and *C. albidus* (*B*) stabilized by 2,6-diaminopimelic acid α-L-rhamnosidases activity on the time of thermal inactivation (pH 5.2; t = 65 °C)**
Here and after * — $P < 0.05$ compared with the control

A comparative study of the activity and stability of native and modified C. *albidus* and *E. erubescens* α-L-rhamnosidases have shown (Fig. 5) that binding with cellulose preparations expedient to carry out at concentrations of 5–15 mg/10 mg of protein. In precisely these concentrations the protective effect on examined enzymes has been observed. Higher concentrations of cellulose derivatives (up to 20–25 μg) resulted in a decrease in thermal stability and complete enzymes inactivation. Thus, the more of binding points enzyme to polymer, the lower its activity. Presumably, the conformational changes that occur in this case prevent the substrate access to substrate-binding and active centers. The greatest activity was observed after 3 h of incubation at 65 °C for *C. albidus* α-L-rhamnosidase modified by MC-cellulose, CM-cellulose (at concentrations of 5–15 μg/10 μg of protein) and Servacel CM 32 at concentration of 15 μg/10 μg of protein. For *E. erubescens* α-L-rhamnosidase, the stabilization by all investigated cellulose preparations except DEAE-cellulose has been significantly influenced.

Modification of certain groups of amino acid residues of the protein by group-specific reagents may also cause significant stabilizing or a destabilizing effect on the enzyme operation. We have previously shown [3, 4], that sulfhydryl groups of cysteine residues are essential for maintaining the active conformation of *E. erubescens* and *C. albidus* α-L-rhamnosidases, as for many other glycosidases. These groups are effective nucleophilic agents with high reactivity. We have calculated the number of SH groups in the molecule, it is equal to $7.2 \cdot 10^{-6}$ mM and $8.64 \cdot 10^{-7}$ mM per mg of protein, respectively, for *E. erubescens* and *C. albidus* α-L-rhamnosidases. Dithiothreitol and cysteine were used as protectors of these groups. But at a certain concentration (10^{-3} M), these substances did not stabilize α-L-rhamnosidases. Histidine and sulfhydryl group modificators DEPC (1 %) and *p*-CMB (10^{-5} M) at studied concentrations accelerated their thermal denaturation (Fig. 6), probably by facilitating the transfer of protein globules to the unfolded state.

Treatment of studied α-L-rhamnosidases with glycine (0.1 M) (Fig. 7) promoted the

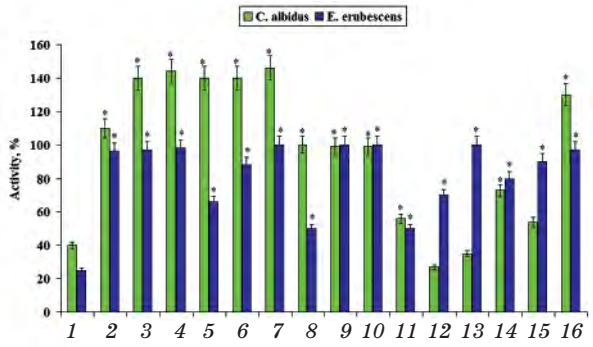

Fig. 5. **Activity of *E. erubescens* and *C. albidus* α-L-rhamnosidases after enzymes modification by different types of cellulose:**
1 — control (native enzyme); *2* — MC-cellulose 5 μg; *3* — MC-cellulose 10 μg; *4* — MC-cellulose 15 μg; *5* — CM-cellulose 5 μg; *6* — CM-cellulose 10 μg; *7* — CM-cellulose 15 μg; *8* — cellulose 5 μg; *9* — cellulose 10 μg; *10* –cellulose15 μg; *11* — DEAE-cellulose 5 μg; *12* — DEAE-cellulose 10 μg; *13* — DEAE-cellulose 15 μg; *14* –Servacel 32 CM 5 μg; *15* –Servacel 32 CM 10 μg; *16* –Servacel 32 CM 15 μg. Incubation — 3 h at 65 °C

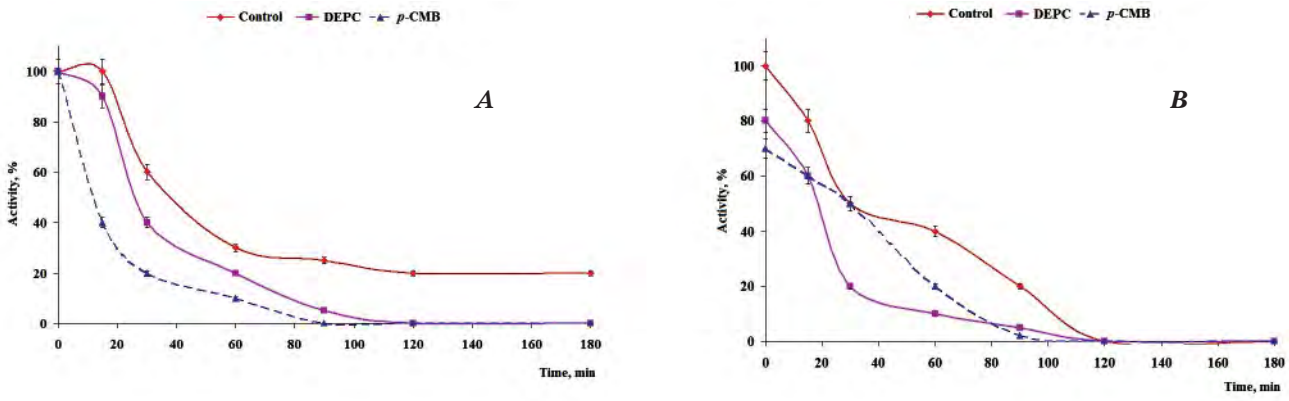

Fig. 6. **Dependence of *E. erubescens* (*A*) and *C. albidus* (*B*) modified α-L-rhamnosidases on the time of thermal inactivation (pH 5.2; t = 65 °C)**

enzyme of *C. albidus* thermal stabilization only for the first two hours of incubation, whereas, by contrast, have caused decreasing of *E. erubescens* α-L-rhamnosidases activity during 60 min treatment. The mechanism of glycine action is, probably, in individual amino acid residues of the protein binding by carboxyl group and amino group of glycine, making it less active conformation.

Thus, the research of *C. albidus* and *E. erubescens* α-L-rhamnosidases modification and stabilization using chemical compounds for thermostable enzyme preparations obtaining has been carried out. It has been shown that in the case of polymers such as PEG 1 500, dextrans 70 T and 500 T usage the thermal stability of investigated α-L-rhamnosidases reduced, while treatment with PEG 20 000 results in thermal stabilization of the enzyme of *E. erubescens* by 280% and *C. albidus* — by 150% (within 180 minutes of incubation). A comparative study of the thermal stability of native and modified by cellulose and its derivatives α-L-rhamnosidases of *C. albidus* and *E. erubescens* indicates that at cellulose

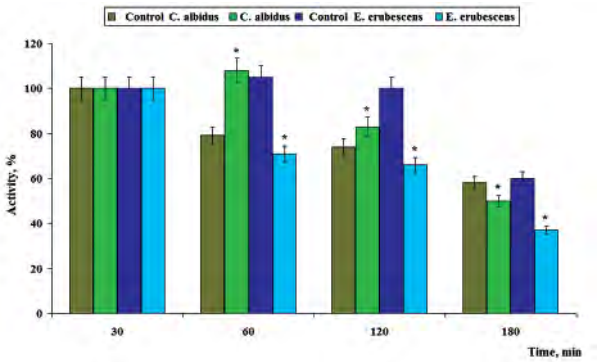

Fig. 7. **The activity of *E. erubescens* and *C. albidus* α-L-rhamnosidases after enzymes modification by 0.1 M glycine**

concentrations of 5–15 μg/10 μg of protein the protective effect on examined enzymes is observed. There may be also an effective approach based on hydrophobic modification of enzymes molecule by acylation of lysine amino groups using succinic anhydride. Based on the obtained data, modified α-L-rhamnosidases of *C. albidus* and *E. erubescens* can be developed for the usage in biotechnological processes.

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ТЕРМОСТАБІЛІЗАЦІЯ α-L-РАМНОЗИДАЗ *Eupenicillium erubescens* ТА *Cryptococcus albidus* ЗА ДОПОМОГОЮ ХІМІЧНИХ РЕАГЕНТІВ

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Метою роботи було порівняльне вивчення термостабільності нативних і модифікованих різними методами α-L-рамнозидаз *Eupenicillium erubescens* і *Cryptococcus albidus* для підвищення їхньої стабільності. Термоденатурацію нативних і модифікованих ензимів проводили за 65 °С, pH 5,2. Активність визначали за допомогою *n*-нітрофеніл-α-L-рамнопіранозиду та нарингіну. Встановлено, що у разі стабілізації поліетиленгліколем 1500, декстранами 70 Т і 500 Т термостабільність досліджуваних α-L-рамнозидаз знижується, тимчасом як використання поліетиленгліколю 20000 сприяє збільшенню термостабільності ензиму *E. erubescens* на 280%, а *C. albidus* — на 150%. Порівняльне вивчення термостабільності нативних і модифікованих целюлозою та її похідними α-L- рамнозидаз *C. albidus* та *E. erubescens* показало, що за концентрацій целюлози 5–15 мкг/10 мкг протеїну спостерігається захисний вплив полімерів на ензими. Гідрофобна модифікація бурштиновим ангідридом також дає змогу в умовах досліду уповільнити термоденатурацію α-L-рамнозидаз. Отримані стабілізовані α-L-рамнозидази *C. albidus* і *E. erubescens* можуть бути використані в біотехнологічних процесах.

Ключові слова: α-L-рамнозидаза, *Eupenicillium erubescens*, *Cryptococcus albidus*, термостабілізація ензимів.

ТЕРМОСТАБИЛИЗАЦИЯ α-L-РАМНОЗИДАЗ *Eupenicillium erubescens* И *Cryptococcus albidus* С ПОМОЩЬЮ ХИМИЧЕСКИХ РЕАГЕНТОВ

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Целью работы было сравнительное изучение термостабильности нативных и модифицированных различными методами α-L-рамнозидаз *Eupenicillium erubescens* и *Cryptococcus albidus* для повышения их стабильности. Термоденатурацию нативных и модифицированных энзимов проводили при 65 °С, pH 5,2. Активность определяли с помощью *n*-нитрофенил-α-L-рамнопиранозида и нарингина. Установлено, что в случае стабилизации полиэтиленгликолем 1500, декстранами 70 Т и 500 Т термостабильность исследуемых α-L-рамнозидаз снижается, в то время как использование полиэтиленгликоля 20000 приводит к увеличению стабильности энзима *E. erubescens* на 280%, а *C. albidus* — на 150%. Сравнительное изучение термостабильности нативных и модифицированных целлюлозой и ее производными α-L-рамнозидаз *C. albidus* и *E. erubescens* показало, что при концентрациях целлюлозы 5–15 мкг/10 мкг протеина наблюдается защитное влияние полимеров на энзимы. Гидрофобная модификация янтарным ангидридом также позволяет в условиях опыта замедлить термоденатурацию α-L-рамнозидаз. Полученные стабилизированные α-L-рамнозидазы *C. albidus* и *E. erubescens* могут быть использованы в биотехнологических процессах.

Ключевые слова: α-L-рамнозидаза, *Eupenicillium erubescens*, *Cryptococcus albidus*, термостабилизация энзимов.