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ACTIVITY AND RELEASE OF ENZYMES EMBEDDED IN POLYVINYL ALCOHOL-BASED CRYOHYDROGELS UNDER –20 °C STORAGE CONDITIONS

Key words: catalase, glucose oxidase, superoxide dismutase, polyvinyl alcohol, cryohydrogels, freeze-thawing.

The enzymes superoxide dismutase (SOD) and catalase (CAT), playing an important role in the antioxidant defense system, as well as glucose oxidase (GOx), which can perform the function of natural non-toxic oxidizers of sugars, are of practical importance for biotechnology and medicine. For even more widespread use of enzymes in practical medicine, it is necessary to ensure the stability of the enzyme, its bioavailability, and to take into account the specificity of its delivery to target tissues. One way to solve this is to embed enzymes in microcarriers, in particular, polyvinyl alcohol (PVA)-based hydrogels.

It is currently emphasized that versatile PVA-based hydrogels can be developed for a wide range of applications in drug delivery, regenerative medicine, wound dressing, water purification, energy storage, *etc.* [3]. Th ere are many methods for preparing PVA-based hydrogels, including cryogelation, which is a cyclic freezing/thawing of PVA in aqueous solutions, with the formation of polyvinyl alcohol cryohydrogels (PVA-C). Obtained by means of cryogelation, which provides optimal mechanical characteristics, high biocompatibility, PVA-C are popular as carriers for biologically active substances and, most importantly, those with controlled release [3]. Moreover, the advantages of the cryogelation are its simplicity, cost-effectiveness and environmental friendliness, which makes it particularly attractive for biomedical applications. The successful incorporation of a protein into a cryogel matrix is determined by the preservation of enzyme activity throughout the time until its use and/or delivery to target tissues and, most importantly, ensuring its controlled release. At the same time, it has been noted that a special attention should be paid to study the long-term stability of the PVA-protein complex due to its impact on both safety and efficiency in medical applications [4].

The aim of this research was to study the effect of a storage of PVA-based cryohydrogels with embedded globular protein enzymes: catalase, superoxide dismutase, glucose oxidase, at –20 °C on the dynamics of protein release from the PVA-C volume and on their catalytic activity.

The study used PVA m.m. 122.5 kDa (Merck, Germany); SOD, CAT and GOx (Sigma-Aldrich Chemical, Germany). The enzyme (100 mg) was dissolved in 10 ml of the appropriate buffer: SOD —

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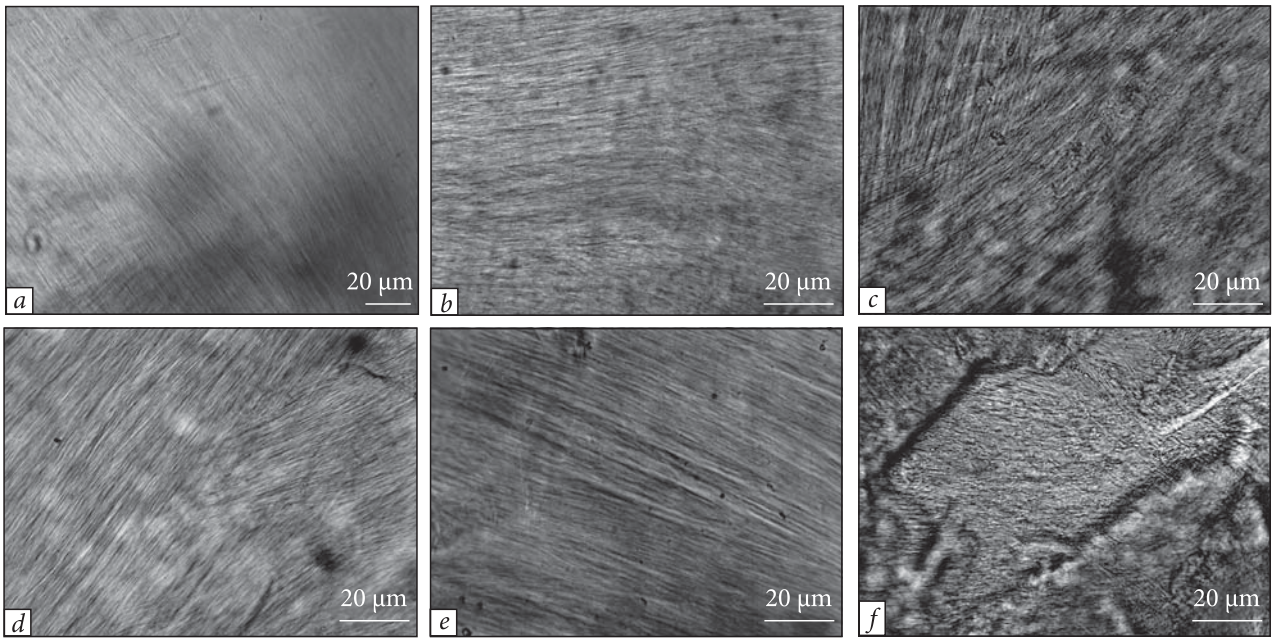

Fig.1. Micrographs of cross-sections of PVA-C surfaces before and after storage at –20 °C for 30 days. Cryohydrogels formed in acetate buffer: *a* — before storage, *d* — after storage; formed in carbonate buffer: *b* — before storage, *e* — after storage; formed in Tris-HCl: *c* — before storage, *f* — after storage

in 0.2 M Na-carbonate buffer, pH = 10.63; CAT — in 0.05 M Tris-HCl buffer, pH = 7.8; GOx — in 0.2 M acetate buffer, pH = 4.7 (initial solutions). To obtain hydrogels, the dry polymer was dispersed in buffer systems appropriate to the enzymes and left to swell for 18 hours at 20 °C. The swollen samples were heated with constant stirring at 90—100 °C until a transparent PVA hydrogel was formed, to which enzyme solutions with a known concentration were added, taking into account the constant final concentration of PVA as 10%. The mixture of hydrogel and protein was placed in a freezer (–20 °C) for 48 hours, then this frozen complex was placed in a refrigerator (4 °C) for 48 hours [5]. In this way, PVA cryohydrogels with embedded proteins were obtained using a single freeze-thaw method. The microstructure of the cryohydrogels was analyzed by examining their thin sections with an AxioObserver Z1 fluorescent confocal microscope (Carl Zeiss, Germany) using polarized light. The width of the "threads" was determined using the AxioVision Rel. 4.8 program (Carl Zeiss, Germany). The enzyme concentration was determined spectrophotometrically by measuring the absorption intensity at a wavelength of 280 nm, characteristic of the protein. The enzyme activity was determined as described [1] in solutions added to the PVA hydrogel during the formation of complexes of PVA-C with proteins — this activity is a control, and in solutions after their release from the complexes before and after their storage. The absorption spectra were recorded with a Pye Unicam SP 8000 spectrophotometer (Pye Unicam Ltd, United Kingdom). To determine the amount of enzyme released from PVA-C, 3 g of cryogel was placed in 5 ml of the appropriate buffer and incubated at 37 °C for 4 hours. To study the effect of sto-rage at –20 °C on the state of globular protein enzy-mes in the composition of PVA-C and on the phy-sical characteristics of PVA-C themselves, the latter were frozen to –20 °C at a rate of 3—5 degrees/min and stored in a freezer for 30 days. Then they were warmed in a water bath at 37 °C until the liquid phase appeared and the enzyme activity in the solutions was measured after their release from the gel. The experimental findings were statistically processed using the "Statistica 6.0" software (StatSoft Inc., USA). The data in the figures are given as the mean value ± standard deviation. The statistical significance of the differences in the studied numerical values was checked by the Mann-Whitney test. Differences were considered significant at $p \leq 0.05$. The number of experiments in each series of experiments was at least five.

Significant characteristics of PVA-C are their porosity and pore size, which affect the degree of swelling of the cryogel and the diffusion of embedded drugs [2]. In this regard, it is important to

assess the effect of the solution composition and repeated freezing of the resulting cryogels during their low-temperature storage on the microstructure of the morphology of PVA-C.

In the micrographs of thin sections of the PVA-C samples (Fig. 1), the surface microstructure is clearly visible, the main elements of which include "threads", long or shorter, with a size in the range from 1.82 to 3.56 μm depending on the composition of the solutions (Fig. 1, *a*, *b*, *c*). After storing PVA-C at –20 °C for 30 days, the main elements practically do not change for the PVA-C formed in acetate and carbonate buffers (Fig. 1, *d*, *e*). Obvious changes in the microstructure occur for the Tris-HCI solution: the "threads" become shorter and chaotically orientated (Fig. 1, *f*), and a narrowing of the "threads" is also observed, which is apparently associated with the concentration of the Tris-HCI solution in the intercrystalline space during freezing. The change in the PVA-C microstructure morphology reflects the densification of the structure that occurs after its storage at low temperature, *i. e.*, additional cryogenic exposure to the formed cryohydrogel. This process is also influenced by the solution composition.

The effect of storage at –20 °C for 30 days on the activity of enzymes embedded in PVA cryohydrogels is shown in Fig. 2.

It can be seen that under the conditions of storage of PVA-C complexes with proteins at – 20 ° C for 30 days, the activity indices of CAT and GOx decreased almost equally and slightly compared to the decrease in SOD activity.

PVA cryogels formed by the physical crosslinking play the role of a skeleton that provides mechanical strength of the system and allows enzymes to be embedded at the formation stage. It was shown that all the studied enzymes are embedded in PVA-C and retain activity at a high level, even after low-temperature storage (Fig. 2).

The release process of the protein-enzymes from the PVA-C volume was studied as follows. The cryohydrogel (3g) with embedded protein before and after storage of the complexes at –20 °C for 30 days was placed in 5 ml of the appropriate buffer and kept at 37 °C for 2 hours; the liquid was drained and the protein concentration in it was measured by spectrophotometry, converting it to its relative amount — protein yield for the first 2 hrs (Fig. 3). Then the cryohydrogel was again filled with buffer, kept at 37 °C for the next 2 hrs and the amount of released protein was determined in the same way —

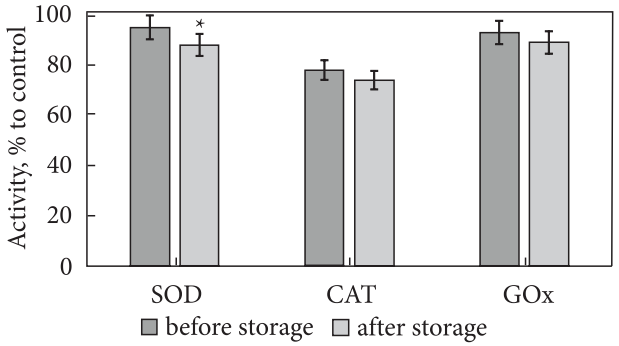

Fig. 2. Enzyme activity in the composition of PVA cryohydrogels before and after storage at –20 °C for 30 days. * — differences are significant compared to SOD activity before storage, $p < 0.05$

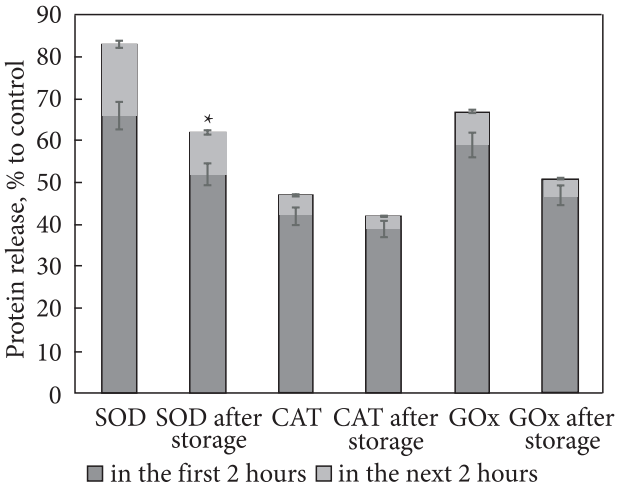

Fig. 3. Protein release before and after storage of PVA cryogel complexes with embedded proteins at –20 °Cfor 30 days. * — differences are significant compared to SOD release before storage, $p < 0.05$

protein yield for the next 2 hrs (Fig. 3). The amount of protein embedded in the PVA-C was assumed as a control. The study showed that the maximum release of enzymes before storage occurs in the first 2 hrs (Fig. 3). During the next 2 hrs of incubation, an additional 18% SOD, 8% GOx, and 5% CAT are released. After storing the enzymes in the composition of PVA-C, the dynamics of their release is kept, but the overall yield decreases, *i. e.*, a slowdown in release is observed, which is most pronounced for SOD (Fig. 3).

There are only a few reported studies that specifically investigate proteins embedded in PVA cryohydrogels. Our results are consistent with those of S. Wang et al. [6], who demonstrated high efficiency of loading bovine serum albumin into nano-particles, PVA and temperature sensitive release profile.

Thus, the effectiveness of the cryogenic method for obtaining a cryohydrogel based on PVA with embedded enzymes (SOD, CAT, GOx) with a high (compared to the original solution) level of activity has been shown. Additional cryogenic effect of moderately low temperatures on the PVA-C complex with enzymes decreases an activity and slows down the release of enzymes from the volume of the cryohydrogel, which depends on the composition of the medium and the embedded protein. The composition of the solution, storage at a temperature of –20 °C (repeated freeze-thawing) affects the structure of the formed PVA-C, which indirectly determines the dynamics of enzyme release from cryogels. A way to better preserve enzymes and optimize their release may be to change the properties of PVA cryohydrogels by altering the number of cycles during their formation and/or using PVA of a different molecular weight.

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АКТИВНІСТЬ ТА ВИВІЛЬНЕННЯ ФЕРМЕНТІВ, ВБУДОВАНИХ У КРІОГІДРОГЕЛІ НА ОСНОВІ ПОЛІВІНІЛОВОГО СПИРТУ, ЗА УМОВ ЗБЕРІГАННЯ ПРИ ТЕМПЕРАТУРІ –20 °С

Ключові слова: каталаза, глюкозооксидаза, супероксиддисмутаза, полівініловий спирт, кріогідрогелі, заморожування-відігрівання**.**