UDC 541.49:546.732/3:547.496.2 doi: https://doi.org/10.15407/ubj96.01.096

MIXED-LIGAND COMPLEXES OF GERMANIUM – 3d-METAL WITH 1-HYDROXYETHANE-1,1-DIPHOSPHONIC ACID AND 2,2′-BIPYRIDINE AS MODULATORS OF *BACILLUS* SP. IMV B-7883 ELASTASE AND FIBRINOGENASE ACTIVITY

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Received: 07 November 2023; **Revised:** 11 December 2023; **Accepted:** 01 February 2024

We have previously shown that Bacillus sp. IMV B-7883 exhibits both elastase and fibrinogenolytic activity. One of the approaches to enhance enzymatic activity is the use of coordination compounds capable of affecting enzyme's activity or synthesis. The purpose of this work was to study the effect of mixed-ligand complexes of ***Ge(IV)*** *– Co(II) (Ni(II), Cu(II)) with 1-hydroxyethane-1,1-diphosphonic acid and 2,2'-bipyridine on the activity of elastase and fibrinogenase purified from Bacillus sp. IMV B-7883. Previously synthesized and characterized mixed-ligand complexes and enzymes purified from the supernatant of the bacterial culture liquid were used in the study. Elastase activity was determined colorimetrically with the use of Congo red, fibrinogenase activity was estimated by fibrinogen hydrolysis measured by absorption at 275 nm. It was shown that complexes* ***1*** *($C_{132}H_{164}Co_4Ge_6N_{24}O_{68}P_{12}$) and* ***2*** *($C_{132}H_{148}Ge_6N_{24}Ni_4O_{60}P_{12}$) inhibited activity of Bacillus sp. IMV B-7883 elastase by 54 and 71% respectively, while complex* ***3*** *($C_{92}H_{128}Cu_4Ge_6N_{16}O_{63}P_{12}$) enhanced it by 30%. Stimulating effect of all three complexes on fibrinogenase activity was revealed. Thus, complex* ***1*** *and* ***2*** *activated the enzyme by more than 50% and complex* ***1*** *– by 19%. The data obtained indicate a complex mechanism of the studied complexes influence on enzymatic activity depending on both their composition and structure.*

K e y w o r d s: elastase; fibrinogenase; Bacillus sp. IMV B-7883; mixed-ligand complexes; germanium – 3d-metal; 1-hydroxyethane-1,1-diphosphonic acid; 2,2'-bipyridine.

In recent years, coordination compounds of various metals have been widely studied by scientists in order to establish their biological activity and pharmacological properties. Thus, various coordination compounds based on such ligands as complexons are used in medicine, biotechnology, optoelectronics, agriculture, etc. That is why one of the most important tasks of modern coordination chemistry is the development and creation of new substances that have properties useful for practice. In this regard, heterometallic complexes of germanium and other metals based on biologically active complexons, such as 1-hydroxyethane-1,1-diphosphonic acid, are of particular interest. It was established that compounds of Ge(IV) with 1-hydroxyethane-1,1-diphosphonic acid (H_4hedp) exhibit hepatoprotective, cardiovascular, anti-hypoxic, detoxifying, and cerebroprotective properties, meaning they have a positive effect on various biochemical processes in the body [1].

Earlier, we showed that complexes of Ge(IV) with H_4hedp are able to play the role of effectors of the synthesis and activity of the following enzymes: collagenase, α-N-acetylgalactosaminidase and α-galactosidase [2].

Since germanium compounds are characterized by low toxicity, they can also be used in the activation of enzymes such as elastase and fibrinogenase, which are important both in medicine and in various industries. So, authors [3] shown that elastase of *Priestia megaterium* gasm32 is not only able to hydrolyze elastin efficiently but also has antibacterial

activity against such well-known antibiotic-resistant bacteria, as *Shigella boydii* ATCC 9207, *Shigella* spp., *Staphyloccocus aureus*. Lei et al. [4] isolated elastase from *Chryseobacterium indologenes* from the mud of a meat market in Ya'an city. Elastase is expected to have potential application in meat tenderization because of its specific hydrolysis toward elastin. Fibrinogenolytic enzymes can be used in the study of fibrinogen/fibrin polymerization processes, and can also be considered as a basis for the creation of drugs aimed at reducing the threat of intravascular thrombus formation by limited proteolysis of fibrinogen circulating in the patient's bloodstream.

We have previously [5, 6] shown that *Bacillus* sp.IMV B-7883 exhibits both elastase and fibrinogenolytic activity. To increase its activity, we chose one of the well-known approaches, in particular, the use of a number of coordination compounds capable of influencing activity of these enzymes. In this regard, the purpose of this work was to study the effect of мixed-ligand complexes germanium – 3d-metal with 1-hydroxyethane-1,1-diphosphonic acid and 2,2′-bipyridine on the elastase and fidrinogenolytic activities of *Bacillus* sp. IMV B-7883.

Materials and Methods

The object of the research was the strain of *Bacillus* sp., isolated from soil and deposited in the Ukrainian Collection of Microorganisms under the number IMV B-7883.

Bacillus sp. IMV B-7883 was submerged cultivated in Erlenmeyer flasks on a liquid medium of the following composition, (g/l): KH_2PO_4 – 1.6; $MgSO_4 \cdot 7H_2O$ – 0.75; $ZnSO_4 \cdot 7H_2O$ – 0.25; $(NH_4)_2SO_4$ – 0.5; maltose – 1.0; gelatin – 10.0; yeast autolysate – 0.15, pH – 7.0. The strain was cultivated for three to six days (72-144 h) in flasks on shaker (100 ml of medium, 28°C, 244 rpm). The inoculum was taken on the same medium for 24 h and inoculated in flasks at a quantity of 105-106 colony-forming units (CFU). Cells were separated from the culture liquid medium by centrifugation at 5000 g for 30 min. The enzymes elastase and fibrinogenase was purified from the supernatant of the culture liquid by precipitation with 90% ammonium sulfate, with further fractionation on neutral and charged carriers, as we described previously [7]. When studying the influence of coordination compounds on the activity of enzymes, final concentrations of 0.1 and 0.01% were used, the ratio of enzyme and coordination compounds was 1:1, exposure time was 1 h and 24 h. After the indicated period of time, aliquots were taken to determine elastase and fibrinogenase activity according to the above methods. In all experiments, enzymatic activity in the absence of compounds (control) was taken 100%.

Elastase activity was determined colorimetrically by the intensity of the color of the solution upon enzymatic hydrolysis of elastin stained with Congo red [8]. The incubation mixture contains 2.5 ml of 0.01 M Tris-HCl buffer (pH 7.5), 5 mg of elastin stained with 0.002% Congo red solution and 1 ml of the cuture liquid. The reaction mixture is incubated for 5 h at a temperature of 37°C. The reaction was stopped by keeping the test tubes with the reaction mixture in an ice bath for 30 min. Unhydrolyzed elastin was separated by centrifugation for 10 min at 10 000 g. The color intensity was measured on an SF-26 spectrophotometer by absorption at 515 nm. The amount of enzyme that catalyzes the hydrolysis of 1 mg of substrate per hour under standard conditions was taken as a unit of elastase activity. Specific elastase activity was 4138 U/mg protein.

To determine fibrinogenolytic activity, fibrinogen was used as a substrate [9]. 1 mg of fibrinogen, 1.8 ml of Tris-HCl buffer (pH 7.5) and 0.2 ml of the studied preparation were added to the test sample. Incubate for 30-45 min at 37°C. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid (TCA). TCA was added to the control sample immediately. Samples were kept at room temperature for 20 min and then centrifuged at 10 000 g for 10 min to remove precipitated protein. Absorption was measured on an SF-26 spectrophotometer at a wavelength of 275 nm. The amount of enzyme that, under the conditions of the experiment, increased absorption by 0.01 in 1 min was taken as a unit of activity.

As modifiers of enzyme activity, we used 3 mixed-ligand complexes of Ge(IV) and Co(II), (Ni(II), Cu(II)) with 1-hydroxyethane-1,1-diphosphonic (H_4hedp) acid and 2,2′-bipyridine (bipy). Their synthesis was carried out as follows. A suspension of GeO_2 (0.1046 g, 1 mmol) and H_4hedp (0.206 g, 1 mmol) in 100 ml of hot water (90°C) was stirred to dissolve reagents completely and then slowly evaporated at 80°C to a 10 ml volume and cooled to room temperature (working solution). Separately, a solution was prepared containing 0.124 g (0.5 mmol) $Co(CH_3COO)_2 \cdot 4H_2O$ (for complex **1**), 0.124 g (0.5 mmol) $Ni(CH_3COO)_2 \cdot 4H_2O$ (for **2**), 0.100 g (0.5 mmol) $Cu(CH_3COO)_2 \cdot H_2O$ (for **3**) and 0.234 g (1.5 mmol) bipy in 10 ml of 95% ethanol,

which was added to the working solution and mixed. After 2 days, crystalline precipitates of compounds **1-3** were formed in the reaction medium. The yield was 62-70%.

Elemental analyses for phosphorus, germanium and other metals were performed using inductively coupled plasma atomic emission spectroscopy with an Optima 8000 PerkinElmer; analyses for C, H and N were performed in Elemental Analyzer CE-440. Thermogravimetric analyses were carried out using a Q-1500D with a heating rate of 10°C/min in air in the temperature range 20-1000°C. The IR absorption spectra of the complexes were collected on a spectrophotometer Frontier PerkinElmer using KBr pellets in the 400–4000 cm^{-1} range. The most important absorption bands in the IR spectra of complexes **1-3** were assigned in compliance with the literature data, including data for the germanium(IV) coordination compounds [10-12].

For the complexes below are the formulas, the results of the elemental analysis – calculated/found (in %), the color and selected IR data:

1 – $C_{132}H_{164}Co_4Ge_6N_{24}O_{68}P_12$ – C 37.57/37.40; H 3.89/3.78; Co 5.60/5.51; Ge 10.34/10.28; N 7.97/7.85; P 8.82/8.76 (yellow). Selected IR data for **1** (in cm^{-1}): 3390 ν(OH), 2921 $\nu(C–H_{Ar})$, 1587, 1518 $\nu(C–C_{Ar})$, 1450, 1426 $\delta_{as}(CH_3)$, 1342 $\delta_s(CH_3)$, 1311 ν(C–N), 1178 ν(P=O), 1048 $\nu_{as}(PO_3)$, 972 $\nu_s(PO_3)$, 848 δ(C–H), 815 δ(Ge–OH), 674 ν(Ge–O).

2 – $C_{132}H_{148}Ge_6N_{24}Ni_4O_{60}P_{12}$ – C 38.83/38.47; H 3.63/3.54; Ge 10.71/10.64; N 8.25/8.15; Ni 5.80/5.73; P 9.14/9.06 (pink). Selected IR data for **2** (in cm^{-1}): 3377 ν(OH), 2913 $\nu(C–H_{Ar})$, 1586, 1518 $\nu(C–C_{Ar})$, 1448, 1425 $\delta_{as}(CH_3)$, 1340 $\delta_s(CH_3)$, 1313 ν(C–N), 1179 ν(P=O), 1050 $\nu_{as}(PO_3)$, 973 $\nu_s(PO_3)$, 848 δ(C–H), 814 δ(Ge–OH), 669 ν(Ge–O).

3 – $C_{92}H_{128}Cu_4Ge_6N16O_{63}P_{12}$ – C 31.29/31.18; H 3.63/3.55; Cu 7.26/7.17; Ge 11.36/11.27; N 6.35/6.28; P 10.54/10.46 (blue). Selected IR data for **3** (in cm^{-1}): 3385 ν(OH), 2926 $\nu(C–H_{Ar})$, 1627 $\delta(H_2O)$, 1580, 1520 $\nu(C–C_{Ar})$, 1451, 1420 $\delta_{as}(CH_3)$, 1342 $\delta_s(CH_3)$, 1309 ν(C–N), 1183 ν(P=O), 1050 $\nu_{as}(PO_3)$, 972 $\nu_s(PO_3)$, 853 δ(C–H), 818 δ(Ge–OH), 668 ν(Ge–O), 486 ν(Cu–O).

Reagents from Sigma-Aldrich were used for the synthesis of compounds: GeO_2, $Co(CH_3COO)_2·4H_2O$, $Ni(CH_3COO)_2·4H_2O$, $Cu(CH_3COO)_2·H_2O$, 1-hydroxyethane-1,1-diphosphonic acid ($C_2H_8O_7P_2$, CAS 7414-83-7), 2,2′-bipyridine ($C_{10}H_8N_2$, CAS 366-18-7).

When studying the effect of various germanium-containing compounds on the activity of enzymes, we used concentrations of 0.1 and 0.01% and time of exposure 1 h and 24 h. The studied compounds were dissolved in 0.1% DMSO.

All experiments were performed in 3-5 replicates. Student's *t*-test was used to perform statistical analysis. The data are presented as mean ± standard error ($M \pm m$) and are considered significant at $P < 0.05$. The results presented in graphs were processed using Microsoft Excel 2007.

Results

New-synthesized complexes represent stable in air crystalline compounds with the molar ratio Ge : hedp : Co(Ni) : bipy = 3:3:2:6 (**1**, **2**) and Ge : hedp : Cu : bipy = 3:3:2:4 (**3**).

The thermal decomposition of complexes showed that this process starts with an endothermic effect in the temperature range of 70–220°C (with a maximum at 100-120°C), which was accompanied by the removal of 20, 14 and 18 water molecules per formula unit. The broad temperature range of this process can say that the complexes contain crystallization and coordinated water molecules. The presence of coordinated water was confirmed only in complex **3** by a pronounced band $\delta(H_2O)$ at 1627 cm^{-1}. The rather high temperature of water removal is due to the formation of a wide system of intermolecular and intramolecular hydrogen bonds, which are characteristic of various metal complexonates [1]. The IR spectrum **1-3** showed a stretching band ν(OH) ~ at 3380 cm^{-1}, stretching vibrations of the Ge–O bond (~ 670 cm^{-1}) and Ge–O–H bending vibrations (~ 815 cm^{-1}). The bands at 1050 cm^{-1} – $\nu_{as}(PO_3)$ and 973 cm^{-1} – $\nu_s(PO_3)$ confirmed deprotonated phosphonic groups are present in structures of complexes.

When analyzing the results of research of complexes **1-3**, it was established that the obtained compounds belong to the cation-anion type. They consist of the hexanuclear cyclic anions $[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-hedp})_6]^{8-}$ (Fig. 1), complex cations $[M(bipy)_3]^{2+}$ (M = Co, Ni), $[Cu(H_2O)(bipy)_2]^{2+}$ (Fig. 2) and crystallization water molecules. Germanium atoms in anion are linked in pairs by two bridging ligands: hydroxo (or oxo) and 1-hydroxyethane-1,1-diphosphonatic. In the cation of complexes **1**, **2**, the octahedral coordination polyhedron of the Co, Ni is formed by the six nitrogen atoms of three 2,2′-bipyridine. The coordination number of Cu atom in complex **3** is five, the polyhedron is formed by the four nitrogen atoms of two molecules of 2,2′-bipyridine and oxygen atom of water molecule. The

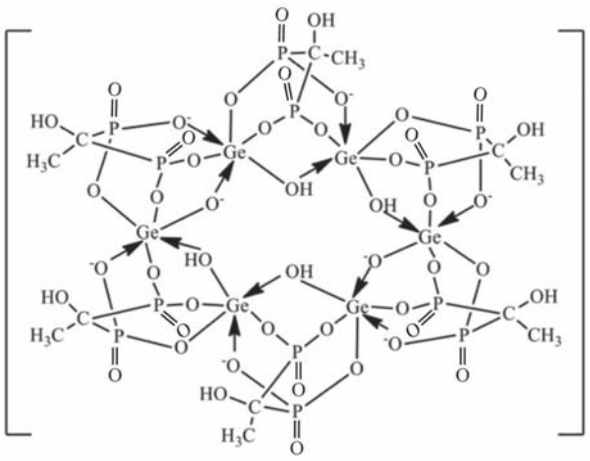

Fig. 1. Scheme of structure of anion $[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-}hedp)_6]^{8-}$

synthesized complexes correspond to the formulas $[M(bipy)_3]_4[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-}hedp)_6]\cdot nH_2O$ (M = Co(**1**), n=20; Ni(**2**), n=12), $[Cu(H_2O)(bipy)_2]_4[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-}hedp)6]\cdot 14H_2O$ **(3)**.

Study of the effect of synthesized compounds on the elastase activity of *Bacillus* sp. IMV B-7883 (Fig. 2, 3) showed that they exhibit diverse effects. Thus, activation (by 30%) of elastase was noted (Fig. 2, *A*, *B*) under the influence of compound **3**. It should be noted that the activating effect did not depend on either the incubation time or the concentration of the test substance. Compounds **1** and **2** inhibited the elastase activity of *Bacillus* sp. IMV B-7883. The degree of inhibition was also practically independent of incubation time and concentration of substances. The greatest inactivation (72%) was observed under the influence of compound 2, while the inhibitory effect of compound **1** was slightly lower (40%).

Study of the effect of substances **1**, **2**, **3** on the protease of *Bacillus* sp. IMV B-7883 with fibrinogenolotic activity showed (Fig. 4, *A, B*) that all compounds exhibited an activating effect. The greatest activation was observed under the influence of compound **1** at a concentration of 0.1% and an exposure time of 1 h (71%) (Fig. 4, *A*). When the action time was increased to 24 h, a decrease in activation by 17% was noted (Fig. 4, *B*). Whereas the activating effect of substance 1 at a concentration of 0.01% remained at the same level regardless of the time of action.

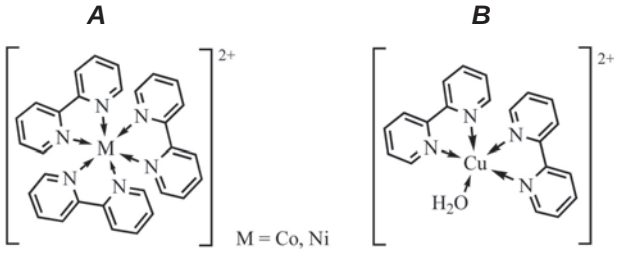

Fig. 2. Scheme of structures of cations $[M(bipy)_3]^{2+}$, $[Cu(H_2O)(bipy)_2]^{2+}$

Compound **2** showed maximum activation (62%) at a concentration of 0.1% and an exposure time of 1 h (Fig. 4, *A*). A decrease in concentration, as well as an increase in action time, led to a decrease (11%) in the activating effect. Compound **3** showed maximum activation (19%) at an exposure time of 24 h (at both concentrations). With a decrease in exposure time, the activating effect of substance **3** was slightly lower (by 15-16%).

Discussion

Enzyme activity can be increased by using various synthetic substances, metal ions or their complex compounds. Among the latter, coordination compounds of active metals, in particular germanium, with bioligands are promising and at the same time least studied [13-15].

The unique complex-forming properties of germanium are used in various fields of medicine and pharmacology, including due to the ability to influence the properties of a protein by changing its conformation [16, 17].

The greatest activation was observed under the influence on elastase activity of compound **3** (30%), and it was at the same level regardless of time and concentration of the substance. A similar pattern is also observed when compounds **1** and **2** inhibit elastase activity (54 and 71 % respectively). Thus, the degree of inhibition did not differ significantly and was approximately at the same level, regardless of time and concentration of substances.

All compounds exhibited an activating effect in relation to the protease of *Bacillus* sp. IMV B-7883 with fibrinogenolytic activity. Complexes **1** and **2** activated the enzyme by more than 50%. The greatest activating effect (71%) was exhibited by compound **1** at a concentration of 0.1% after 1 h of exposure, and the least activating effect (15-19%) was demonstrated by complex **3** regardless of exposure time and concentration of the substance.

Complexes, in terms of their effect on elastase activity, showed to be effectors of different types:

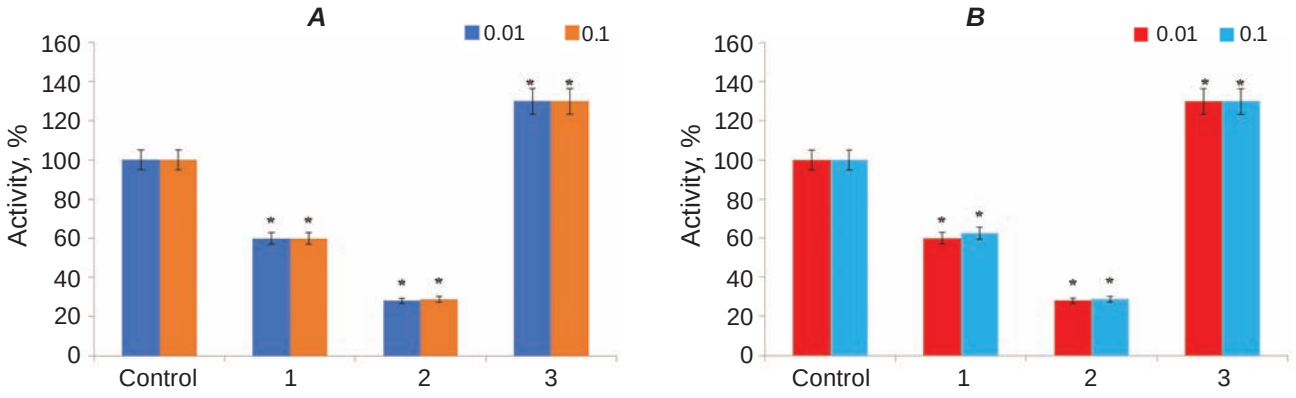

Fig. 3. Influence of germanium compounds 1, 2 and 3 on the elastase activity of Bacillus sp. IMV B-7883. ***A*** *– exposure time 1 h,* ***B*** *– exposure time 24 h. *P < 0.05 as compared to the matched control*

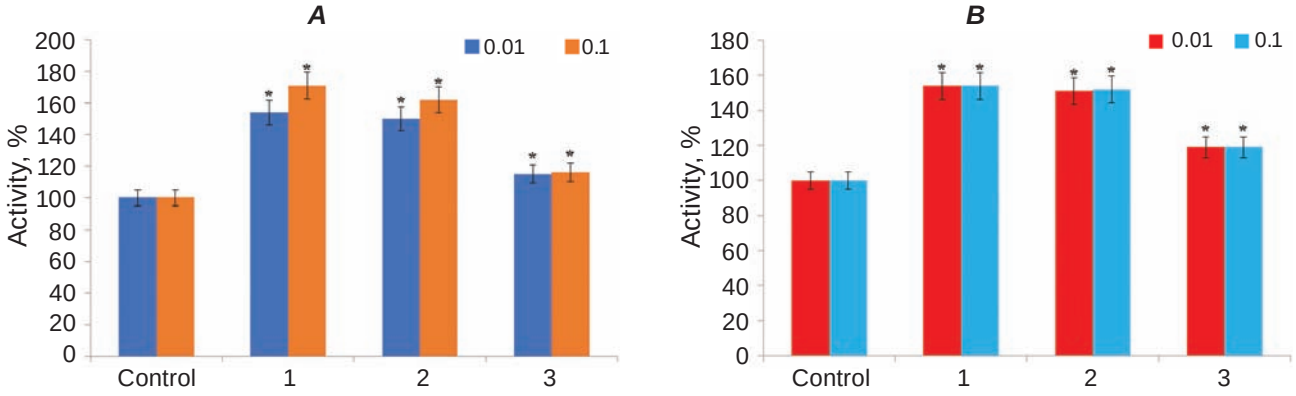

Fig. 4. Influence of germanium compounds 1, 2 and 3 on the fibrinogenolytic activity of Bacillus sp. IMV B-7883. ***A*** *– exposure time 1 h,* ***B*** *– exposure time 24 h. *P < 0.05 as compared to the matched control*

1 and **2** are inhibitors, **3** is an activator. In cases **1** and **2**, competitive inhibition of the enzyme action is observed, part of it is spent on the formation of the enzyme-inhibitor complex, while the amount of enzyme-substrate decreases. Since in the molecules of the complexes $[M(bipy)_3]_4[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-}hedp)_6]\cdot nH_2O$ (M = Co(**1**), n = 20; Ni(**2**), n = 12) the composition and structure of the complex anions are the same, the cations are similar, we can conclude that it is the replacement of Co with Ni that influences the increase in inhibition. The composition $[Cu(H_2O)(bipy)_2]_4[Ge_6(\mu\text{-}OH)_4(\mu\text{-}O)_2(\mu\text{-}hedp)_6]\cdot 14H_2O$ (**3**) contains a cation that is completely different in composition and structure. Its influence, on the contrary, leads to the activation of the substrate and many functional groups of the enzyme, to their more complete conformational correspondence.

It is typical that when studying the fibrinogenolytic activity of the enzyme, complexes **1-3** showed themselves equally as activators according to the series **3**<**2**<**1**.

The data obtained indicate a complex mechanism of action of effectors depending on both their composition and the structure and nature of the enzyme.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Funding. Research was carried out within the framework of the state budget topic “Secondary metabolites of microorganisms with antibiotic, proteolytic and glycolytic action: patterns of macromolecule organization, functional and biological activity, ways of modification and stabilization, aspects of practical use” (state registration number 0123V102324).

ЗМІШАНОЛІГАНДНІ КОМПЛЕКСИ ГЕРМАНІЮ – 3d-МЕТАЛІВ З 1-ГІДРОКСИЕТАН-1,1-ДИФОСФОНОВОЮ КИСЛОТОЮ ТА 2,2′-БІПІРИДИНОМ ЯК МОДУЛЯТОРИ ЕЛАСТАЗНОЇ ТА ФІБРИНОГЕНАЗНОЇ АКТИВНОСТІ *BACILLUS* SP. IMV B-7883

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Раніше ми показали, що *Bacillus* sp. IMV B-7883 проявляє як еластазну, так і фібриногенолітичну активність. Одним із підходів до підвищення ензиматичної активності є використання координаційних сполук, здатних впливати на активність або синтез ензиму. Метою даної роботи було вивчення впливу різнолігандних комплексів Ge(IV) – Co(II), (Ni(II), Cu(II)) з 1-гідроксиетан-1,1-дифосфоновою кислотою та 2,2′-біпіридином на активність еластази та фібриногенази, очищеної з *Bacillus* sp. IMB B-7883. У дослідженні використовувалися раніше синтезовані та охарактеризовані змішанолігандні комплекси та ензими, очищені з супернатанту культуральної рідини бактерії. Активність еластази визначали колориметрично з використанням конго червоного, фібриногеназну активність оцінювали за гідролізом фібриногену. Показано, що комплекси **1** ($C_{132}H_{164}Co_4Ge_6N_{24}O_{68}P_{12}$) і **2** ($C_{132}H_{148}Ge_6N_{24}Ni_4O_{60}P_{12}$) пригнічують активність еластази *Bacillus* sp. IMV B-7883 на 54 і 71% відповідно, тоді як комплекс **3** ($C_{92}H_{128}Cu_4Ge_6N_{16}OO_{63}P_{12}$) посилює її на 30%. Виявлено стимулюючу дію всіх трьох комплексів на фібриногеназну активність. Так, комплекс **1** і **2** активували ензим більш ніж на 50%, а комплекс **3** – на 19%. Отримані дані свідчать щодо складного механізму впливу досліджуваних комплексів на ензиматичну активність залежно як від їх складу, так і від структури.

Ключові слова: еластаза; фібриногеназа; *Bacillus* sp. IMV B-7883; змішанолігандні комплекси; германій – 3d-метал; 1-гідроксиетан-1,1-дифосфонова кислота; 2,2′-біпіридин.

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