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INFLUENCE OF NEW TYPES OF BISCITRATOGERMANATES ON *PENICILLIUM RESTRICTUM* α-L-RHAMNOSIDASE

The intensive development of biotechnology in the last decade is largely determined by the growing requirement needs of both medicine and various industries for products of microbial synthesis, including glycosidases, in particular α-L-rhamnosidases. Their wide use to solve current biological-medical and chemical-technological problems stimulates researchers to search for compounds capable of influencing their catalytic activity. Therefore, the ***purpose*** *of this work was to isolate and purify α-L-rhamnosidase from a new producer of Penicillium restrictum and to investigate multi-ligand germanium-3d-metal complexes with citric acid, phenanthroline, and bipyridine as effectors of its activity.* ***Methods.*** *The object of the study was α-L-rhamnosidase of P. restrictum. Its purification was carried out by gel filtration and ion exchange chromatography on TSK-gels and Sepharose 6B. The activity of α-L-rhamnosidase was determined using the Davis method with naringin as a substrate. As modifiers of enzyme activity, purposefully synthesized multiligand germanium-3d-metal complexes with citric acid, phenanthroline, and bipyridine ($[Ni(bipy)_3][Ge(HCit)_2]\cdot 3H_2O$ (**1**); $[Ni(phen)_3][Ge(HCit)_2]\cdot 2H_2O$ (**2**); $[\{Cu(bipy)_2\}_2Ge(\mu\text{-}Cit)_2]\cdot 12H_2O$ (**3**); $[\{Cu(phen)_2\}_2Ge(\mu\text{-}Cit)_2]\cdot 13H_2O$ (**4**); $[Zn(bipy)_3][Ge(HCit)_2]\cdot 2H_2O$ (**5**); $[Zn(phen)_3][Ge(HCit)_2]\cdot 3H_2O$ (**6**)), were used.* ***Results.*** *From the supernatant of culture fluid of P. restrictum, α-L-rhamnosidase was isolated and purified 23.1 times with a yield of 0.09%. The specific activity of the enzyme was 27.8 units/mL. The enzyme was homogeneous according to gel filtration on Sepharose 6B and had a molecular mass of 50 kDa. It was established that the considered coordination compounds are able to regulate the catalytic activity of α-L-rhamnosidase of P. restrictum. All of them manifest themselves either as activators or as inert substances, no inhibition was observed. In addition, the dependence of the degree of enzyme activation by*

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the compounds on their concentration is traced and corresponds to the following series: at a concentration of 0.01% — 1 > 6 ≈ 5 > 3 >2 ≈ 4 and at a concentration of 0.1% — 1 > 4 > 2 > 5 ≈ 6.3. The catalytic activity is also significantly affected by the time of exposure to the compounds: at a concentration of 0.01% for 1h, the activity of the enzyme at the control level was observed for all compounds, whereas at a concentration of 0.1% for 24 h,the activity increased sharply in the presence of compounds 1 (300%), 6 (153%), and 2 (134%). The action of the others was at the control level. ***Conclusions.*** *The obtained data on new complex metal compounds with an activating effect on microbial α-L-rhamnosidases. It has been established that compounds whose structural organization ensures the synergism of the action of all components are the most promising enzyme effectors in a series of coordination compounds of biologically active metals and ligands.*

Keywords: *Penicillium restrictum, α-L-rhamnosidase, multi-ligand germanium-3d-metal complexes with citric acid, phenanthroline, and bipyridine.*

In the plant world, a group of biologically active substances, flavonoids, derivatives of benzo-γ-pyrone, the basis of which is a phenylated skeleton, is widespread. In plants, flavonoids are rarely found in the free state, most of them are represented by glycosides, which are cleaved into monosaccharides (L-rhamnose, D-glucose, D-galactose, D-xylose, L-arabinose) and aglycones under the action of glycosidases [1]. One of the leading roles among glycosidases is played by α-L-rhamnosidase (EC 3.2.1.40), which hydrolytically splits out the terminal α-1,2-, α-1,3-, and α-1,4-linked residues of L-rhamnose, present in both synthetic and natural glycosides, glycolipids, oligosaccharides, and various conjugates, derivatives of flavonoids, such as rutin, neohesperidin, naringin, and quercetin. They are characterized by various types of pharmacological effects, including cardiological, antitumor, hypotensive, antispasmodic, and a number of others. For the manifestation of the biological effect by some of them, the presence of rhamnose in their composition is necessary, while for the others — its absence [1—3].

Earlier [5, 6], we isolated a number of active producers of α-L-rhamnosidases. But since the enzymes of different producers are characterized by various properties, it is necessary to search for new producers of α-L-rhamnosidases, as well as to study the influence of a number of synthetic compounds on their activity. Therefore, the purpose of this work was to isolate and purify α-L-rhamnosidase from a new producer of *Penicillium restrictum* and to investigate multi-ligand germanium-3d-metal complexes with citric acid, phenanthroline, and bipyridine as effectors of its activity. The choice of these coordination compounds was determined by the presence in their composition of all biologically active components such as essential germanium, metals of life (Ni, Cu, Zn), citric acid (a participant in the Krebs cycle), as well as reagents used in chemistry, biology. and medicine such as 1, 10-phenanthroline and 2,2'-bipyridine [7].

Materials and methods. The object of the study was α-L-rhamnosidase of *Penicillium restrictum*. Isolation of the enzyme was carried out by fractionation with ammonium sulfate: dry salt was added to the culture liquid supernatant up to 90% saturation under pH control. The mixture was kept for 24 h at 4 °C and centrifuged (2.5 × g for 30 min). The precipitate was collected, dissolved in 3 volumes of 3 M ammonium sulphate, and added with 0.01 M sodium azide for preservation. The methods of ion exchange and gel permeation chromatography were used to purify the enzyme. The sediment obtained after fractionation with ammonium sulphate was centrifuged, dissolved in 1.5 volumes of the appropriate buffer, pH 6.0—7.5. The samples were subjected to gel filtration on a column (1.8 × 50 cm) with neutral TSK gel — Toyopearl HW-55 («Toson», Japan), equilibrated with 0.01 M phosphate buffer, pH 6.0. Elution was carried out at a rate of 20 mL/h. Ion exchange chromatography was performed on a column (2.5 × 40 cm) with Toyopearl DEAE-650 (M) («Toson», Japan), equilibrated with 0.01 M Tris-HCl buffer pH 7.5. The sample

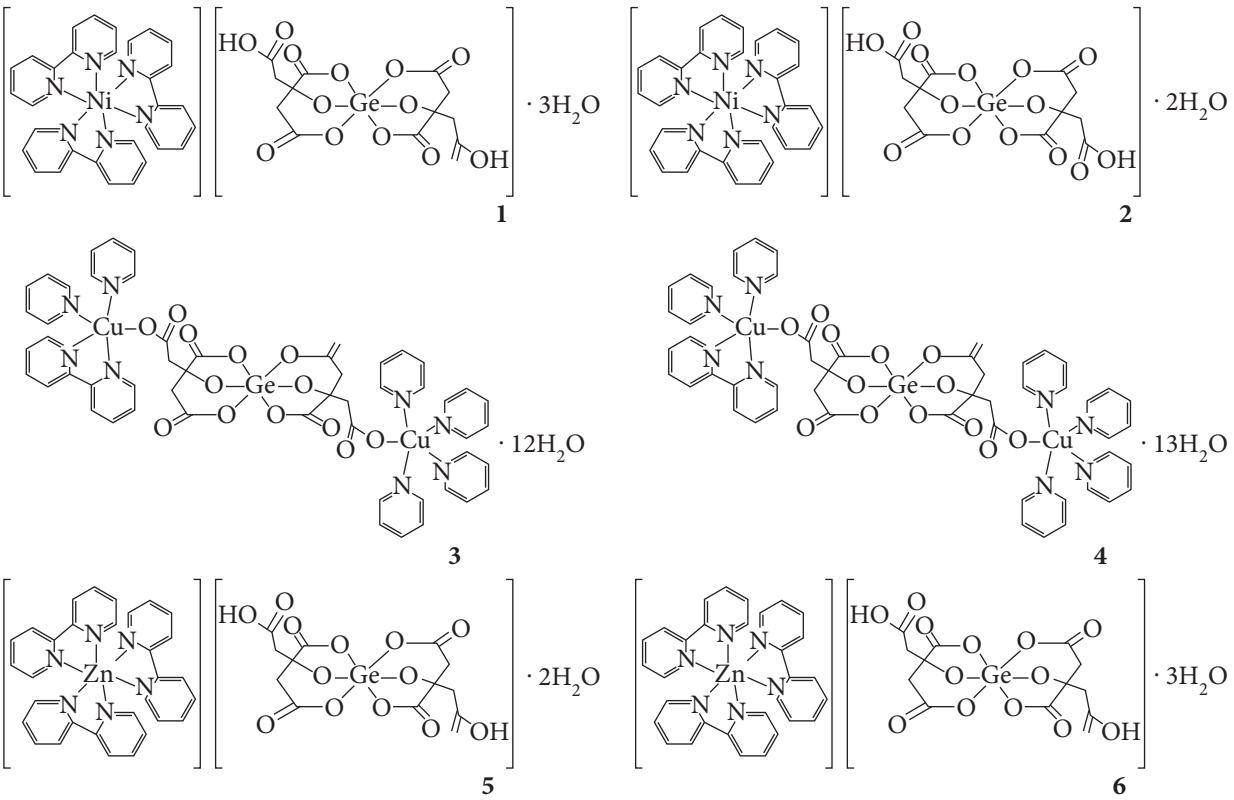

Fig. 1. Schemes of compound structures (1—6)

(about 100 mg of protein) was applied to the column, and elution was carried out with the same buffer in a linear NaCl gradient (0-1 M, 200 mL each) at a rate of 30 mL/h. Fractions showing enzymatic activity were collected, combined, and concentrated under vacuum ≈ 5 times.

Rechromatography was performed on a column (1.3 × 52 cm) with Sepharose 6B, equilibrated with 0.01 M phosphate buffer, pH 6.0. Elution was carried out with the same buffer with 0.1 M NaCl at a rate of 60 mL/h. 1 mL of sample (20 mg of protein) was applied to the column. Protein content was recorded on a DeNovix DS-11 spectrophotometer/fluorimeter, and fractions containing enzymatic activity were collected, combined, and concentrated (≈ 10 times) by evaporation under vacuum.

The activity of α-L-rhamnosidase was determined using the Davis method [8] with minor modification. The assay mixture contained 0.2 mL of 0.1% naringin (Sigma, USA) solution in 0.1 M PCB pH 5.2 and 0.2 mL l enzyme solution. After incubation at 37 ºC for 30 min, the reaction was stopped by the addition of 5 mL diethylene glycol (90%) and 0.1 mL of 4 N NaOH. The residual naringin was measured at 420 nm. One unit of α-L-rhamnosidase activity was defined as the amount of enzyme that re1eases 1 μmol of naringin per min in the solution.

To modify the enzymatic activity, purposefully synthesized multi-ligand germanium-3d-metal complexes with citric acid, phenanthroline, and bipyridine were used for the first time: $[Ni(bipy)_3][Ge(HCit)_2]\cdot 3H_2O$ (**1**); $[Ni(phen)_3][Ge(HCit)_2]\cdot 2H_2O$ (**2**); $[\{Cu(bipy)_2\}_2Ge(\mu\text{-}Cit)_2]\cdot 12H_2O$ (**3**); $[\{Cu(phen)_2\}_2Ge(\mu\text{-}Cit)_2]\times$ $\times 13H_2O$ (**4**); $[Zn(bipy)_3][Ge(HCit)_2]\cdot 2H_2O$ (**5**); $[Zn(phen)_3][Ge(HCit)_2]\cdot 3H_2O$ (**6**), where phen is 1,10-phenanthroline, bipy is 2,2'-bipyridine, and H_4Cit is citric acid.

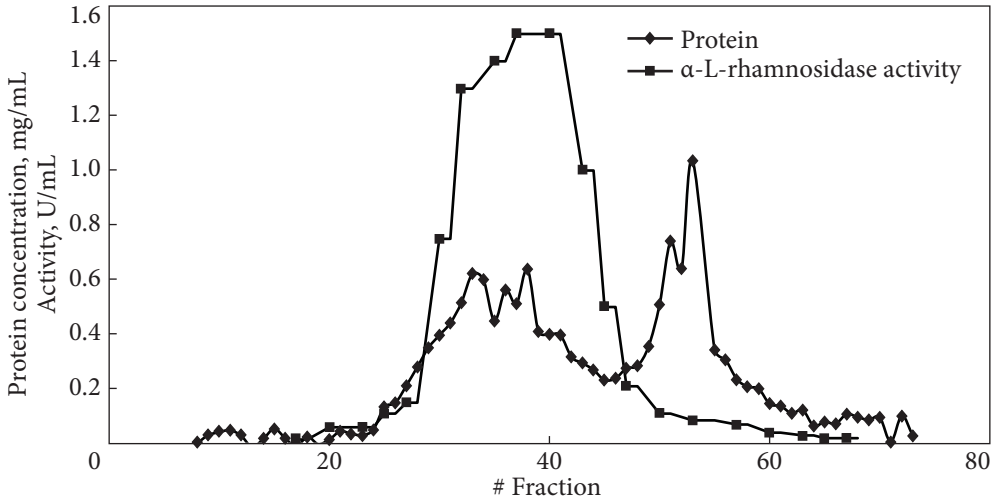

Fig. 2. Elution profile of *P. restrictum* enzyme preparation on TSK-HW-55 (pH 6.0)

All compounds were characterized by a set of data of modern research methods such as elemental analysis, thermogravimetry, and IR spectroscopy. Their crystal structures have been deposited in the Cambridge Structural Data Bank (CCDC 1854034 (2), 1914329 (3), 2020641(4), 2020643 (6)) [9—11]. Schemes of compound structures are shown in Fig. 1.

Table 1. **Purification of *P. restrictum* α-Lrhamnosidase**

Purification steps	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield, %	Degree of purification
Culture liquid supernatant (0.5 L)	450	550	1.2	100	1
90% ammonium sulphate	390	500	1.3	86	1.1
TSK-gel Toyopearl HW-55	120	264	2.2	26	1.8
Toyopearl DEAE-650M	20	171	8.57	4.4	7.14
Rechromatography Sepharose 6B	4.3	120	27.8	0.09	23.1

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

All experiments were carried out in seven replicates. Their results were analyzed through statistical processing using Student's t-test. In the work, mean values and standard errors (M ± m) were calculated. Values at $p < 0.05$ were considered significant. Their results presented graphicallywere processed using Microsoft Excel 2007.

Results. From the supernatant of the culture liquid *P. restrictum,* α-L-rhamnosidase was isolated and purified (by 23.1 times) by gel permeation and ion exchange chromatography with a yield of 0.09% (Table 1, Figs. 2—4). The specific activity of the enzyme was 27.8 units/mL. The enzyme was homogeneous according to gel filtration on Sepharose 6B and had a molecular mass of 50 kDa (Figs. 4—5).

The study of the influence of coordination compounds of germanium (1—6) on the activity of α-L-rhamnosidase of *P. restrictum* showed (Fig. 6, *a*, *b*) a diverse effect. In most cases, the activity of the enzyme was higher when exposed to compounds in a concentration of 0.1%. Under the experiment conditions,

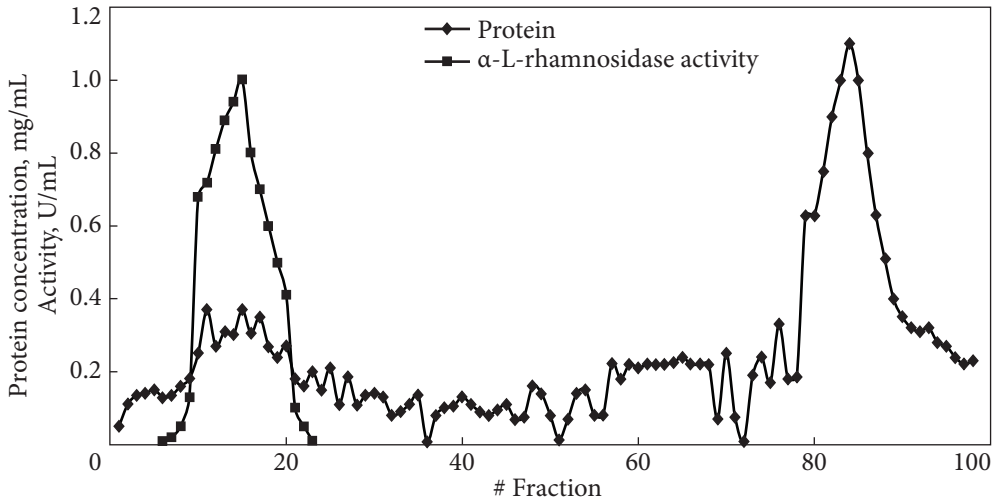

Fig. 3. Elution profile of the *P. restrictum* enzyme preparation on Toyopearl DEAE-650M in a 0-1 M NaCl gradient

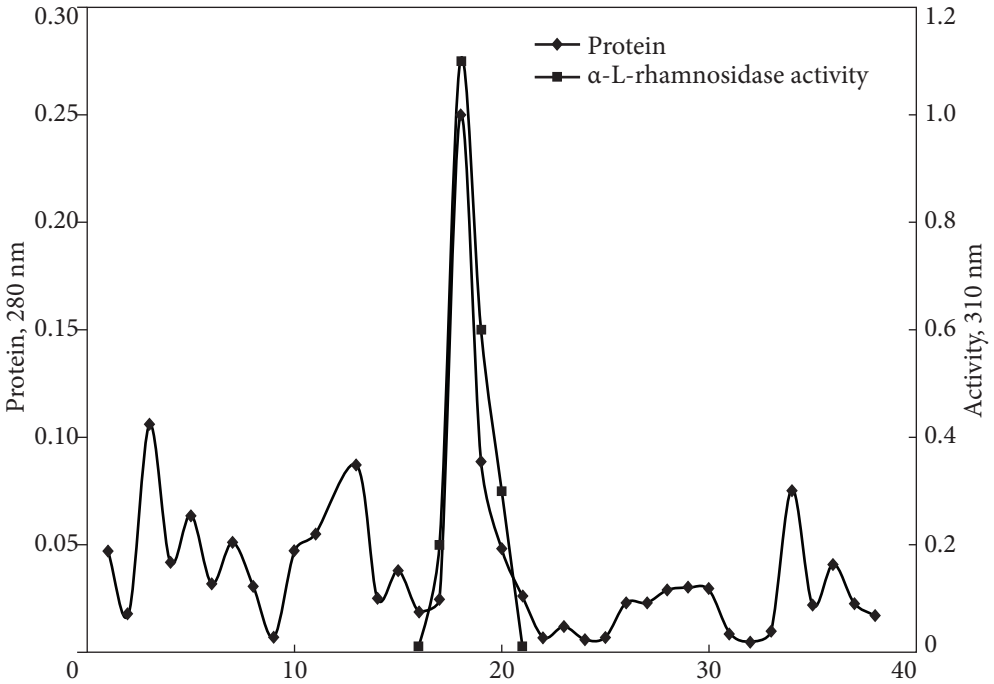

Fig. 4. Rechromatography on Sepharose 6B α-L-rhamnosidase of *P. restrictum*

the activity of *P. restrictum* α-L-rhamnosidase increased by 3 times in the presence of compound 1 at a concentration of 0.1% and an exposure time of 24 h (Fig. 6, *b*), while with an exposure of 1 h, it increased by 2.18 times. The effect of substance 1 at a concentration of 0.01% was less significant. An activity increase by 25% was observed during the first-hour incubation (Fig. 6, *a*), and then it decreased to 16% after exposure for 24 h (Fig. 6, *b*). Compounds 2, 3,

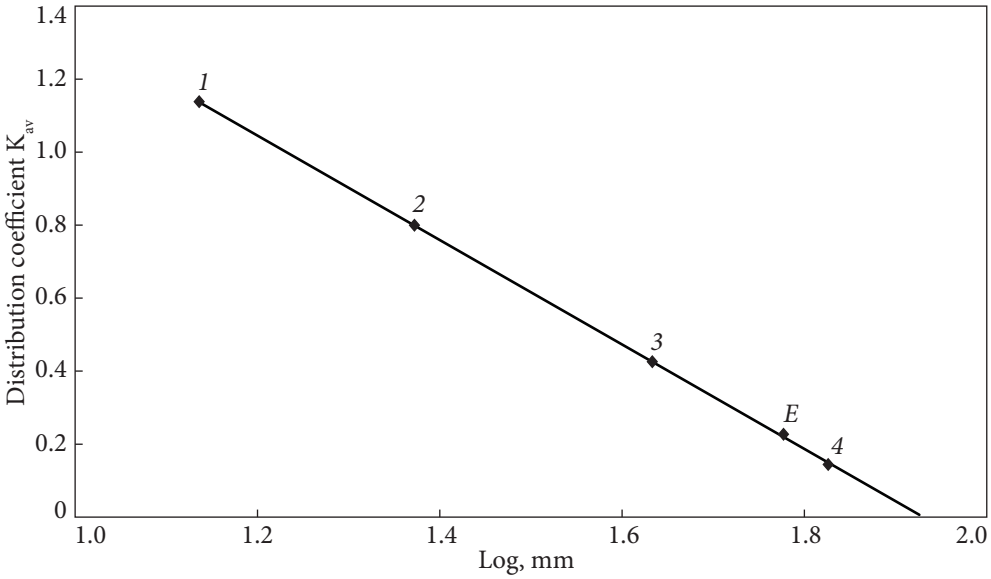

Fig. 5. Molecular mass determination for *P. restrictum* α-L-rhamnosidase in the native system. Molecular markers: (1) ribonuclease (13.7 kDa), (2) proteinase K (25 kDa), (3) chicken ovalbumin (43 kDa), (4) bovine serum albumin (67 kDa), (E) purified enzyme

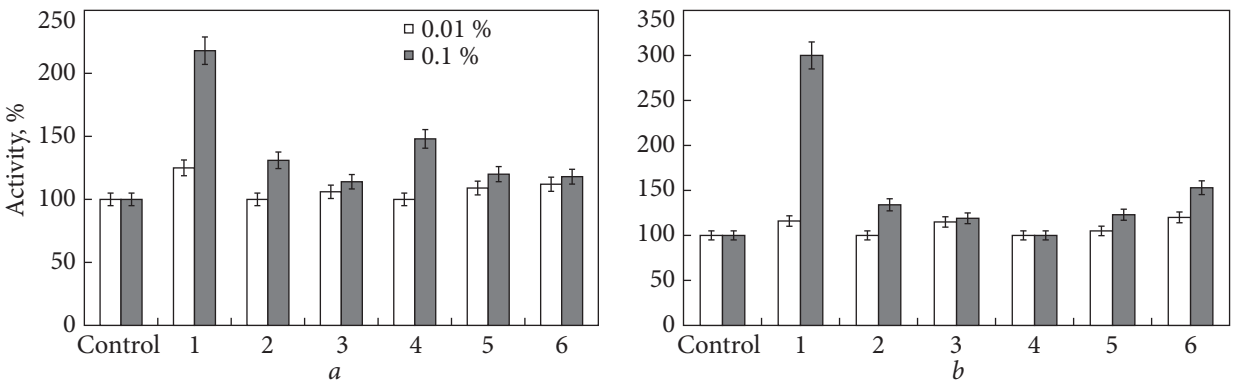

Fig. 6. Influence of germanium compounds on the activity of *P. restrictum* α-L-rhamnosidase: *a* — exposure time 1 h, *b* — exposure time 24 h

5, and 6 at a concentration of 0.1% increased the *P. restrictum* α-L-rhamnosidase activity by 14—53%, depending on the experimental conditions. At a lower concentration (0.01%), compounds 3, 5, and 6 activated the enzyme only by 6—20% (Fig. 6, *a*, *b*).

Under the action of compounds 2 and 4 at a concentration of 0.01%, the activity was at the control level (Fig. 6, *a*, *b*). It is worth noting that compound 4 increased the activity of the studied enzyme by 48% only at a concentration of 0.1% and exposure for 1 h. In other cases, the activity of the enzyme under the action of substance 4 was at the control level.

Discussion. The intensive development of biotechnology in the last decade is largely determined by the growing needs of both medicine and various industries for products of mi-

crobial synthesis, which include glycosidases, in particular α-L-rhamnosidases. The wide use of them to solve current biological-medical and chemical-technological problems stimulates researchers to search for compounds capable of influencing their catalytic activity. Coordination compounds of some metals, in particular germanium, are among such compounds [12—16]. Previously, we have noted both the activating and inhibitory effect of complex compounds of germanium and its dioxide on the synthesis and activity of a number of glycosidases and proteases of microorganisms [17, 18]. But it should be noted that the effect of such compounds on proteins is difficult to predict because of the complexity and variability of their structure. An important role can also be played by the competing reaction of complexation with the germanium ion and formation of multi-metal and multi-ligand complexes, especially in the case of metalloenzymes.

It has been established that the considered coordination compounds are able to regulate the catalytic activity of α-L-rhamnosidase of *P. restrictum*. All of them manifest themselves either as activators or as inert substances; no inhibition was observed. In addition, the dependence of the degree of enzyme activation by the compounds on their concentration is traced and corresponds to the following series: at a concentration of 0.01% — $1 > 6 \approx 5 > 3 > 2 \approx 4$ and at a concentration of 0.1% — $1 > 4 > 2 > 5 \approx 6{,}3$.

The catalytic activity is also significantly affected by the time of exposure to the compounds: at a concentration of 0.01% for 1 h, the activity of the enzyme at the control level was observed for all compounds, and when exposed for 24 h and at a concentration of 0.1%, it increases sharply in the presence of compound 1 (300%), 6 (153%), and 2 (134%), while the action of the others was at the control level.

Enzymatic catalysis belongs to biospecific reactions, which in relation to reagents are considered in the chain composition-structure-properties-function. The studied coordination compounds are biscitratogermanates with phenanthroline/bipyridine complexes of Ni^{2+}, Cu^{2+}, and Zn^{2+}. This allows us to trace the dependence of their influence on the catalytic activity of the enzyme depending on the substitution of the 3d metal and heterocyclic amine (phen or bipy). At the same time, it is important to pay attention to the crystal structure of the complexes. Coordination compounds belong to two types: 1, 2, 5, and 6 are double complexes of the cation-anion type, whereas 3 and 4 are trinuclear multi-ligand-heterometallic complexes (Fig. 1).

Establishing the mechanism of enzymatic catalysis is usually a difficult task [19]. However, based on the obtained data regarding the general characteristics of the studied effectors, it can be stated that the effect on the activity of the enzyme is exerted by all components of complex molecules in total as it varies depending on the compound.

It is known that the leading role in the mechanism of enzymatic catalysis is played by enzyme-substrate complexes, in which the conformation of the enzyme changes reversibly, which contributes to the best spatial correspondence of the enzyme and substrate molecules [19]. Effectors facilitate its formation and reduce the reaction activation energy due to preliminary preparation of the substrate and convergence of functional groups in the most favorable position for the reaction.

The effect of the studied complexes on the catalytic activity of the enzyme that we found is consistent with the one presented above. Thus, complex 1 was the most effective, in which the cation and anion do not compete due to opposite charges, but are bound to various reactive groups of the protein and (or) substrate, that is, they are synergists. Compared to complex 1, structurally similar compounds 2, 5, and 6 were less active for various reasons, namely, as a result of replacing nickel with zinc, or bipyridine with phen-

anthroline. The nickel ion is more reactive and bipyridine is less bulky and more spatially accessible compared to phenanthroline. Complexes 3 and 4 are heteronuclear, their reactive centers are partially blocked, which reduces the activity of these compounds as effectors.

Thus, it can be stated that the most promising way is the search for new enzyme effectors in a series of coordination compounds of biologically active metals and ligands, the structural organization of which ensures the synergism of the action of all components.

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ВПЛИВ НОВИХ ТИПІВ БІСЦИТРАТОГЕРМАНАТІВ
НА АКТИВНІСТЬ α-L-РАМНОЗИДАЗИ *PENICILLIUM RESTRICTUM*

Інтенсивний розвиток біотехнології в останнє десятиріччя в значній мірі визначається зростаючими потребами як медицини, так і різних галузей промисловості в продуктах мікробного синтезу, до яких відносяться глікозидази, зокрема α-L-рамнозидази. Широке використання їх для вирішення актуальних біолого-медичних та хіміко-технологічних проблем стимулює дослідників до пошуку сполук, здатних впливати на їхню каталітичну активність. **Метою** даної роботи було виділити та очистити α-L-рамнозидазу з нового продуцента *Penicillium restrictum* та дослідити різнолігандні германій-3d-метальні комплекси з лимонною кислотою, фенантроліном, біпіридином як ефекторами її активності. **Методи.** Об'єктом дослідження була α-L-рамнозидаза *Penicillium restrictum*. Для очистки ензиму проводили гель-фільтрацію та іонообмінну хроматографію на ТСК-гелях та Sepharose 6B. Активність α-L-рамнозидази визначали за методом Девіса з використанням нарингіну як субстрату. Як модифікатори активності ферментів використано цілеспрямовано синтезовані мультилігандні комплекси германій-3d-метал з лимонною кислотою, фенантроліном, біпіридином, а саме: ($[Ni(bipy)_3][Ge(HCit)_2]\cdot 3H_2O$ (**1**); $[Ni(phen)_3][Ge(HCit)_2]\cdot 2H_2O$ (**2**); $[\{Cu(bipy)_2\}_2Ge(\mu\text{-}Cit)_2]\cdot 12H_2O$ (**3**); $[\{Cu(phen)_2\}_2Ge(\mu\text{-}Cit)_2]\cdot 13H_2O$ (**4**); $[Zn(bipy)_3][Ge(HCit)_2]\cdot 2H_2O$ (**5**); $[Zn(phen)_3][Ge(HCit)_2]\cdot 3H_2O$ (**6**)). **Результати.** Із супернатанту культуральної рідини *P. restrictum* отримали α-L-рамнозидазу, очищену у 23,1 рази, з виходом 0,09%. Питома активність ферменту становила 27,8 од/мл. Фермент був гомогенним за даними гель-фільтрації на Sepharose 6B і мав молекулярну масу 50 кДа. Встановлено, що розглянуті координаційні сполуки здатні регулювати каталітичну активність α-L-рамнозидази *P. restrictum*. Усі вони проявляють себе або як активатори, або як інертні речовини, гальмування не спостерігається. Крім того, простежується залежність ступеня активації ферменту сполуками від їхньої концентрації, що відповідає наступному ряду: за концентрації 0,01% — 1 > 6 ≈ 5 > 3 > 2 ≈ 4; за концентрації 0,1% — 1 > 4 > 2 > 5 ≈ 6,3. На каталітичну активність також суттєво впливає час експозиції сполук: протягом години та при концентрації 0,01% активність ферменту на контрольному рівні спостерігали для всіх сполук, а при експозиції протягом 24 год і концентрації 0,1% активність різко зростає в присутності сполук 1 (300%), 6 (153%), 2 (134%), дії інших були на рівні контролю. **Висновки.** Отримані дані про нові комплексні сполуки металів з активуючою дією на α-L-рамнозидазу. Встановлено, що найбільш перспективними ефекторами ферментів у ряді координаційних сполук біологічно активних металів і лігандів є сполуки, структурна організація яких забезпечує синергізм дії всіх компонентів.

Ключові слова: *Penicillium restrictum, α-L-рамнозидаза, мультилігандні комплекси германій-3d-метал з лимонною кислотою, фенантроліном і біпіридином.*