IMPACT OF THIOSEMICARBAZONE [Cu(H_2L)Cl] COORDINATION COMPOUND ON ACID AND NEUTRAL PROTEASES FROM *Trichoderma koningii* CNMN FD 15 STRAIN

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Abstract. The influence of [Cu(H_2L)Cl] complex on proteolytic activity of *T. koningii* Oudem. CNMN FD 12 fungal strain was evaluated during the 7-11 days of cultivation. The stimulatory effect of the concentrations of 5-15 mg/L of metal complex on the activity of neutral and acid proteases was observed. The peak of acid and neutral protease activity was determined on the 9th and 10th day of cultivation, similar to the control sample. The most favorable concentration of [Cu(H_2L)Cl] was 10 mg/L. Following purification of the proteases by gel filtration resulted in an increase of the specific activity of the control preparation of 1.3-1.7 times for neutral proteases and 1.3-1.8 times for acid proteases. The overall yield, determined for fractions with proteolytic activity, constituted 19% for neutral proteases, and 21% for acid proteases. Purification of the optimized preparation showed an increase in specific activity of the neutral protease of 0.8-1.3 times and of the acid proteases of 1.5-1.7 times. The overall yield was 12.2% for neutral proteases and 18.8% for acid proteases. SDS-PAGE electrophoresis of isolated proteins from the chromatographic fractions showed polypeptide bands only in proteolytic active fractions. Some new polypeptides with apparent molecular mass of 51 and 49 kDa appeared in the polypeptide profile of optimized preparation. The changes were most likely induced by the utilization of [Cu(H_2L)Cl] coordination compound in the *T. koningii* cultivation process.

Keywords: *Trichoderma koningii*; protease; coordination compound; enzyme purification; SDS-PAGE.

INTRODUCTION

Hydrolytic enzymes, including proteases, also called industrial enzymes, are of enormous practical value, being widely used in various technological processes, medicine and agriculture. Enzymes are part of most industrial catalytic processes. Proteases are extensively used in medicine, in fine chemical technologies (limited proteolysis), in the textile, leather, paper and food industries, to the production of detergents and concentrate feeds in zootechny [12, 23, 26].

In particular, enzymes of microbial origin, derived from bacteria, molds and yeasts, are used [2, 24]. More widely used as sources of extracellular hydrolase are mycelial fungi. The major protease producers are fungi from genera *Aspergillus, Rhizopus, Fusarium*, *Mucor*, *Trichoderma* [17]. The ability of micromycetes to secrete enzymes in the culture medium, to grow on cheap media prepared from food processing by-products and to produce wide range of proteolytic enzymes, active in a large pH range (4-11) gives them extra biotechnological importance [11].

The biosynthetic processes in the fungal cell can be relatively easy regulated by varying of the nutritive factors and cultivation conditions [19, 25].

A relative new direction in the stimulation and regulation of microbial biosynthesis is the utilization of coordination compounds of transition metals. These compounds are characterized by high physiological activity conditioned by the large number of chemical elements, including microelements and numerous active groups of atoms from the composition and by theirs complex structures, similar to natural metallic compounds [16, 21]. The effect of the coordination compounds is expressed by the significant increase of biosynthesis of the bioactive substances, by the reduction of the technological cycle of the microorganism and by the obtaining of final products with predicted composition [14].

Numerous studies have revealed the classes of the coordination compounds with positive effect on the growth and development of microorganisms from different taxonomic groups [3, 27].

From the coordination compounds of transition metals with a broad spectrum of biological activity on mycelial fungi were distinguished the complexes of cobalt (II, III), copper (II), zinc (II), iron (II, III) and nickel (II) with organic ligands of different chemical classes - amino acids, dioximes, thiosemicarbazides, thiocarbamides, thiosemicarbazones [4, 9, 10].

Thiosemicarbazones are known as promising compounds among Schiff bases due to their interesting chemical and structural properties. They are remarkable interest thanks to the co-presence of nitrogen and sulfur atoms in thiosemicarbazide fragment, the elements, that are present in biological systems such as metalloproteins and metalloenzymes and that coordinate and modulate the redox properties of transition metal ions. Thus, thiosemicarbazones are important group of multidentate organic molecules with potential binding sites available for a wide variety of metal ions and with perspective to improve biological properties on complexation. The structural diversity and wide range of biological properties is related to the diversity of ligands possible to obtain by Schiff reaction, varying different parts of the thiosemicarbazide moiety, the set of donor atoms, the different parent carbonyls (aldehydes or cetones). At the same time, the copper(II) ion is considered to be a biometal with the greatest potential among transition metals, with a high chelating capacity and positive

redox potential that allows its participation in biological transport reactions [15]. Copper is one of the most abundant metallic elements in biological systems. In enzymology, copper is somewhat similar to iron in that it function in a series of oxidases, oxygenases and low-molecular-weight electron transfer proteins that are reminiscent of ferredoxins. Furthermore, one class of superoxide dismutases contains copper, as well as polyphenoloxidases and tyrosinases. Copper is a bioactive metal required for growth of microorganisms since it is a cofactor for numerous enzymes [22].

From this point of view, the aim of the research was to evaluate the impact of new copper (II) complex of the composition [Cu(H_2L)Cl], (where H_2L - is a monodeprotonated form of thiosemicarbazone ligand derived from 2-hydroxy-3-carboxy-1-naphthaldehyde) on acid and neutral proteases from *Trichoderma koningii* CNMN FD 15 strain.

MATERIAL AND METHODS

Microorganism and culture conditions

Object of study, micromycete *Trichoderma koningii* Oudem. CNMN FD 15 – producer of acid (pH 3.6), neutral (pH 7.4) and alkaline (pH 9.2) proteases, isolated from the soil of central area of Moldova, is stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Republic of Moldova [13]. The strain was cultivated in Erlenmeyer flasks of 1000 mL volume, at temperature of 28-30 °C, on shakers (180-200 rpm), during 7-11 days. Each flask contains 200 mL of nutrient medium of the following, chosen as optimal, composition (g/L): wheat bran - 20.0; soy flour - 10.0; $(NH_4)_2SO_4$ – 1.0; $CaCO_3$ – 2.0 and [Cu(H_2L)Cl] in concentration of 1.0-20.0 mg/L. Metal complex was added to the autoclaved cultivation medium in the form of solution, prepared at dissolving 100 mg of the compound in 12 mL dimethylformamide and filled to 100 mL with sterile distilled water. Initial nutrient medium pH was 6.25. As control served variants of *T. koningii* CNMN FD 15 grown standard, without coordination compound.

Spore suspension in quantity of 10% v/v with density $1 \cdot 10^6$ spores/mL, obtained by washing with sterile water a 12-14 days culture grown on malt-agar oblique columns was used as seed material.

Coordination compound [Cu(H_2Nthios)Cl] (1)

Copper (II) complex **1** derived from 4-formyl-3-hydroxy-2-naphthoic acid and thiosemicarbazide was for the first time synthesized in Institute of Chemistry, MECR of Republic of Moldova. Additional information about the synthesis and properties of the coordination compound **1** can be found in the annex.

Enzyme extraction

The medium was separated from biomass by filtration and centrifuged 20 minutes at 4000-6000 rpm (centrifuge Nüve NF 800). The proteases were isolated from supernatant with 96% ethanol cooled to -15 °C in ratio 1:4 C_2H_5OH, pH of sedimentation: 8, sedimentation duration: two hours. The sediment was centrifuged 20 minutes at 6000 rpm and dried at 20-22 °C. Obtained enzyme preparation was further investigated and purified.

Enzyme assay

Activity of proteases was determined in the cultural liquid of micromycete during cultivation and in obtained enzyme preparations according to the modified Anson method [1] using as a substrate of sodium caseinate for neutral proteases (pH 7.4) and hemoglobin for acid proteases (pH 3.6). Enzymatic activity unit (U/mL or U/g) was considered the amount of enzyme which for 1 minute at 30 °C transforms the substrate proteins into peptides corresponding to 1 μM Tyr (1 μM Tyr = 0.181 mg). 2 mL of 2% buffered substrate (sodium caseinate/hemoglobin dissolved in the buffer solution with identical volumes of 0.1 M acetic acid, 0.1 M phosphoric acid and 0.1 M boric acid solution adjusted to the necessary pH with 1 M NaOH solution) and 2 mL of adequately diluted enzyme sample was incubated 10 minutes at 30 °C. The reaction was stopped with 4 mL of 0.3 M trichloroacetic acid. After 20 minutes of incubation at 30 °C, the reaction mixture was filtrated through filter paper. 1 mL of filtrate was mixed with 5 mL of 0.5M sodium carbonate solution and 1 mL of diluted 2 M Folin's reagent in ratio 1:2. The absorbance of the samples was measured after 20 minutes at 630-670 nm against control samples. The succession of the reagents was inversely in control samples: 2 mL of enzyme solution was inactivated with 4 mL trichloroacetic acid and after 10 minutes incubation at 30 °C was mixed with 2 mL substrate. The next steps were the same as for experimental samples.

Protein determination

Protein was estimated according to the Lowry method [20] using Bio-Rad DC Protein Assay Kit, taking crystalline bovine serum albumin as the standard. Protein concentration was measured on UV-Visible Spectrophotometer T60.

Proteases purification

Purification of the enzyme preparations was performed by gel filtration: 100 mg of dried enzyme preparation was washed with 1 mL of 20 mM TRIS-HCl buffer (pH 7.5); centrifuged for 10 minutes at 14000 rpm (centrifuge 5415C eppendorf). The supernatant (extract) was collected and loaded onto Toyopearl gel filtration column (14 mL volume). Column equilibration and protein elution was performed with the same buffer; the flow rate was 1 mL/min. The amount of protein and enzyme activity was determined. Active fractions were collected.

Gel electrophoresis

The proteases purity was checked by SDS-PAGE (15%), carried out by Laemmli method [18]. Gel was stained with coomasie Brilliant Blue R-250. The SDS-PAGE molecular weight markers were Phosphorylase b (97.4 kDa), Bovine serum albumin (67.0 kDa), Egg albumin (45.0 kDa), Carbonic anhydrase (29.0 kDa) and Trypsin inhibitor (21.0 kDa).

Statistical analysis

Three replicates of each sample were used for statistical analysis. Data are displayed as mean ± SEM. Statistical analysis was conducted by Student's t test. A p≤0.05 was considered statistically significant.

RESULTS

The influence of [Cu(H_2L)Cl] coordination compound on proteolytic activity of *T. koningii* CNMN FD 15 fungal strain was evaluated in dynamics, during the 7-11 days of cultivation (Fig. 1). Earlier studies showed the maximal exoproteases biosynthesis by *T. koningii* in the 9-10 days grown culture [13].

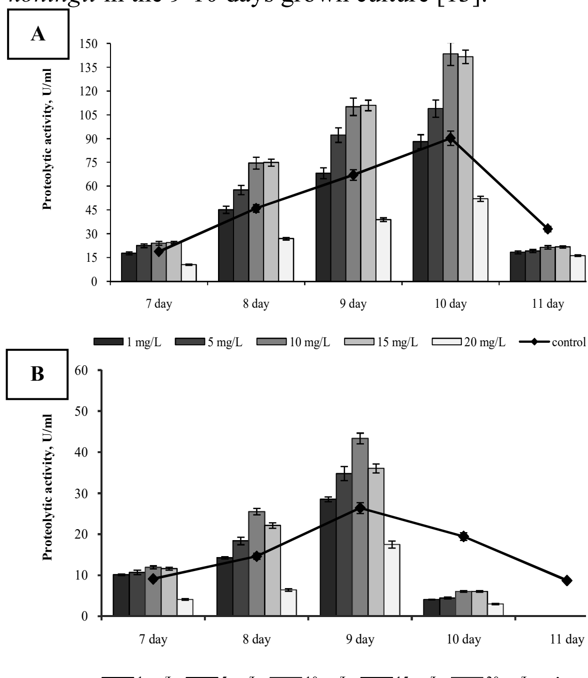

Figure 1. Dynamics of the activity of neutral (A) and acid (B) proteases of *T. koningii* CNMN FD 15 cultivated in the presence of [Cu(H_2L)Cl] coordination compound during 7-11 days.

It was observed the stimulatory effect of the concentrations of 5-15 mg/L of metal complex on the activity of both neutral and acid proteases. The peak of acid protease activity was determined on the 9th day of cultivation and the peak of neutral protease activity – on the 10th day of cultivation, similar to the control sample. The most favorable concentration of [Cu(H_2L)Cl] was 10 mg/L. The acid protease activity increased with 64.18%, being 43.36 U/mL and the activity of neutral protease activity increased with 58.89%, being 143.46 U/mL, compared to the control of the same day (26.41 U/mL on the 9th day for acid protease and 90.29 U/mL on the 10th day for neutral protease). A significant increase in the activity of neutral proteases was obtained on the 9th day of cultivation. The neutral protease activity was 110.10 U/mL, with 64.10% higher compared to the control sample of the same day (67.09 U/mL) and with 21.94% higher compared to the control sample of the 10th day (90.29 U/mL), the day with the maximal activity of neutral proteases.

Thus, for further investigations we have chosen the cultural liquid from the 9th day grown culture of *T. koningii* CNMN FD 15 which showed the maximal activity of acid proteases (43,36 U/mL) and high activity of neutral proteases (110.10 U/mL). Following sedimentation with 96% ethanol of the enzymes from cultural liquid has ensured the obtaining of new enzyme preparation with activity of neutral and acid proteases.

In the table 1 are presented the particularities of the obtained enzyme preparations from the *T. koningii* CNMN FD 15 micromycete cultivated in the standard conditions (control) and under directed conditions with the supplementation of the [Cu(H_2L)Cl] metallocomplex. The protein content in both preparations was identical, the total and specific enzyme activity is, however, higher in the optimized preparation.

The purification profiles of acid and neutral proteases from the control and optimized preparation are presented in Tables 2 and 3.

Purification of the proteases by gel filtration resulted in an increase of the specific activity of the control preparation of 1.3-1.7 times for neutral proteases and 1.3-1.8 times in the case of acid proteases. The overall yield, determined for fractions with proteolytic activity, constituted 19% for neutral proteases, and 21% for acid proteases.

Purification of the optimized preparation showed an increase in specific activity of the neutral protease of 0.8-1.3 times and of the acid proteases of 1.5-1.7 times. The overall yield was 12.2% for neutral proteases and 18.8% for acid proteases.

Although the purification fold of both types of proteases was greater for the control preparation, the total and specific enzyme activity remained significantly higher for the optimized preparation. The activity of neutral proteases of the optimized preparation in the fraction with the most significant enzyme activity (fraction 4) was 3.6 times higher compared to the control preparation and the specific activity was 4.2 times higher for the same fraction (Table 2). The activity of acid proteases of the

Table 1. Proteolytic properties of enzyme preparations obtained from *T. koningii* CNMN FD 15

Variants	Protein (%)	Type of the enzyme	Enzyme activity (U/g)	Specific activity (U/mg)
T. koningii CNMN FD 15 grown under standard conditions	24.73	Neutral proteases	427.92	1.73
		Acid proteases	307.70	1.24
T. koningii CNMN FD 15 grown with 10mg/L [Cu(H_2L)Cl] supplementation	24.46	Neutral proteases	2353.53	9.62
		Acid proteases	1162.82	4.75

optimized preparation in the fraction 4 with the most significant enzyme activity was 3.4 times higher compared to the control preparation. For the specific activity of acid proteases was observed differences in the fractions. Thus, the most significant specific activity of the optimized preparation was in the fraction 3, being 3.6 times higher compared to the most significant specific activity of control preparation observed in the fraction 4 (Table 3).

The lower purification fold of proteases from the optimized preparation was conditioned by the lower initial impurity of the optimized preparation with proteins of a different nature than the proteases, in comparison with the control preparation. The initial specific activity of neutral proteases and acid proteases was 5.5 times and, respectively, 3.8 times higher in the optimized preparation compared to the control (Table 1).

Elution profile of the proteases and protein content in the separated 8 fractions by gel filtration (Fig. 2A) from the control preparation showed the separation of 3 fractions with activity of neutral proteases (fractions 2-4) and 3 other fractions with the activity of acid proteases (fractions 3-5).

SDS-PAGE electrophoresis of isolated proteins from the chromatographic fractions demonstrated the presence of the polypeptides in active fractions 2-5. The polypeptide bands repeated the polypeptide spectrum of the preparation extract, and the most significant polypeptides showed the apparent molecular masses of 77.5; 47 and 26 kDa (Fig. 2B). Different was the elution profile of optimized preparation proteins compared to the control preparation (Fig. 3A). The activity of neutral and acidic proteases was determined in the same fractions (fractions 3-5).

Table 2. Purification profile of neutral proteases (pH 7.4) from proteolytic preparations obtained from *T. koningii* CNMN FD 15

Purification steps	Protein (mg/mL)	Proteolytic activity (U/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
control					
Proteolytic preparation solution (100 mg/mL)	24.73	42.79	1.73	100.0	1.0
Extract	9.68	21.25	2.19	49.7	1.3
Toyopearl gel filtration column					
fraction 2	0.54	1.21	2.24	2.8	1.3
fraction 3	1.12	3.14	2.80	7.3	1.6
fraction 4	1.26	3.80	3.00	8.9	1.7
[Cu(H_2L)Cl]					
Proteolytic preparation solution (100 mg/mL)	24.46	235.35	9.62	100.0	1.0
Extract	12.26	123.85	10.09	52.6	1.0
Toyopearl gel filtration column					
fraction 3	0.80	7.11	8.85	3.0	0.9
fraction 4	1.09	13.92	12.70	5.9	1.3
fraction 5	0.98	7.84	7.92	3.3	0.8

Table 3. Purification profile of acid proteases (pH 3.6) from proteolytic preparations obtained from *T. koningii* CNMN FD 15

Purification steps	Protein (mg/mL)	Proteolytic activity (U/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
control					
Proteolytic preparation solution (100 mg/mL)	24.73	30.77	1.24	100.0	1.0
Extract	9.68	20.25	2.09	66.0	1.7
Toyopearl gel filtration column					
fraction 3	1.12	2.13	1.90	6.9	1.5
fraction 4	1.26	2.24	2.24	9.2	1.8
fraction 5	0.92	1.51	1.63	4.9	1.3
[Cu(H_2L)Cl]					
Proteolytic preparation solution (100 mg/mL)	24.46	116.28	4.75	100.0	1.0
Extract	12.26	71.61	5.83	61.6	1.2
Toyopearl gel filtration column					
fraction 3	0.80	6.54	8.15	5.6	1.7
fraction 4	1.09	7.76	7.08	6.7	1.5
fraction 5	0.98	7.53	7.61	6.5	1.6

SDS-PAGE electrophoresis of isolated proteins from the chromatographic fractions showed polypeptide bands only in proteolytic active fractions (Fig. 3B). The polypeptide profile showed differences, from that observed for the control preparation. Some new polypeptides with apparent molecular mass of 51 and 49 kDa appeared and the 77.5 kDa polypeptide present in the control was not detected. However, some obvious similarities have been observed, the apparent molecular weight polypeptide of 26 kDa has also been identified in the optimized preparation. This changes obtained in the optimized preparation were most likely induced by the utilization of $[Cu(H_2L)Cl]$ coordination compound in the *T. koningii* cultivation process. Coordination compounds induce significant, multiple changes in the proteins of various organisms, regardless of their nature: structural, enzymatic etc. [6].

DISCUSSION

Coordination compounds of the metals are widely synthesized in the recent years and their biological effect is intensely investigated. The studied metal complex of copper (II) derived from 4-formyl-3-hydroxy-2-naphthoic acid thiosemicarbazone ligand $[Cu(H_2L)Cl]$ confirmed earlier researches and demonstrated visible stimulatory effect on enzyme activity of neutral and acid proteases by *T. koningii* CNMN FD 15 micromycete in the concentrations of 5-15 mg/L on the 9th-10th day of cultivation. Obtained positive results allow us to recommend $[Cu(H_2L)Cl]$ complex as stimulator and regulator of neutral and acid protease synthesis by *T. koningii* CNMN FD 15.

It is still less known about the mechanism of influence of metal complexes on enzyme synthesis of microorganisms and mycelial fungi, in particular. The addition of metal ions to nutritive media induces various modifications in proteins structure and their functions. For the first time this research shows that $[Cu(H_2L)Cl]$ coordination compound has an indirect effect on acid and neutral proteases synthesis by *T. koningii* CNMN FD 15 micromycete. The SDS-PAGE electrophoresis of the proteins isolated from the enzyme preparations obtained from *T. koningii* CNMN FD 15 demonstrated visible differences in the polypeptide spectrum of preparation obtained with

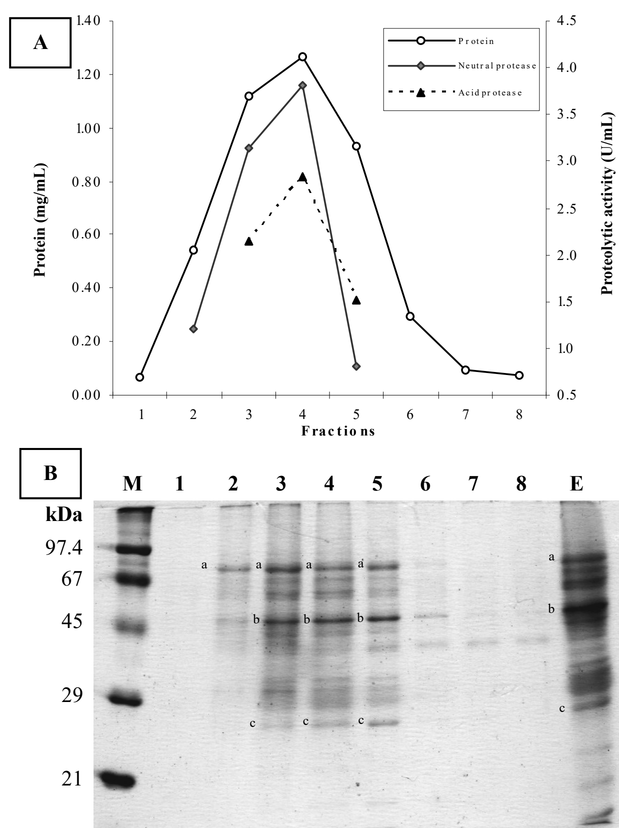

Figure 2. Purification of proteases from control preparation by gel filtration (A). Sample: 1 mL of protease preparation after washing with 20 mM TRIS-HCl buffer (pH 7.5) and centrifuging for 10 minutes at 14000 rpm. Column: Toyopearl gel-filtration column (14 mL volume). Flow rate: 1 mL/min. Buffer: 20 mM TRIS-HCl, pH 7.5; SDS-PAGE electrophoresis of proteases from control preparation of *T. koningii* CNMN FD 15 (B). Lane M: Molecular weight marker, Lane 1-8: Gel filtration samples, Lane E: Extract of preparation sample. [a] 77.5 kDa, [b] 47 kDa, [c] 26 kDa.

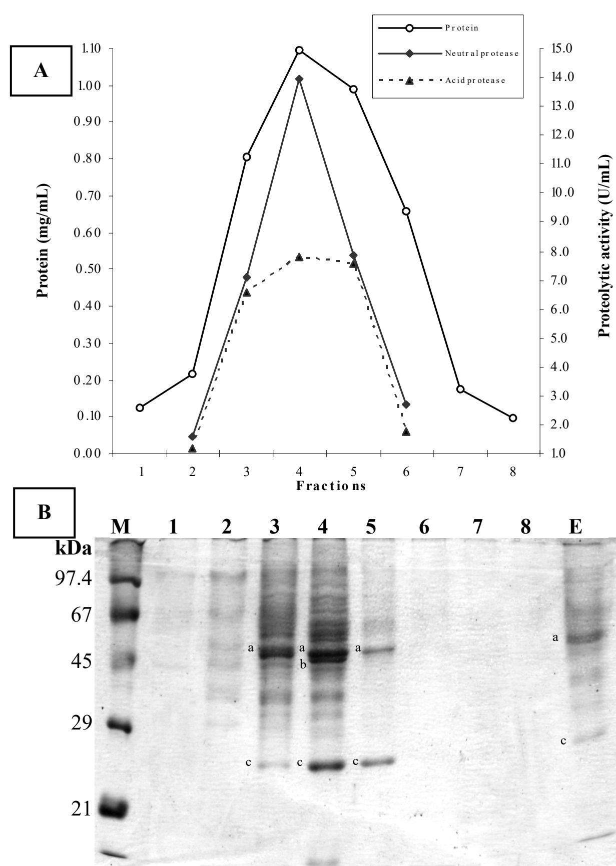

Figure 3. Purification of proteases from optimized preparation by gel filtration (A). Sample: 1 mL of protease preparation after washing with 20 mM TRIS-HCl buffer (pH 7.5) and centrifuging for 10 minutes at 14000 rpm. Column: Toyopearl gel-filtration column (14 mL volume). Flow rate: 1 mL/min. Buffer: 20 mM TRIS-HCl, pH 7.5; SDS-PAGE electrophoresis of proteases from optimized preparation of *T. koningii* CNMN FD 15 (B). Lane M: Molecular weight markers, Lane 1-8: Gel filtration samples, Lane E: Extract of preparation sample. [a] 51 kDa, [b] 49 kDa, [c] 26 kDa.

supplementation of [Cu(H_2L)Cl] in the micromycete growth medium. The changes in the polypeptide spectrum of the proteolytic active fractions eluted through gel filtration chromatography allow us to suppose that [Cu(H_2L)Cl] is direct involved in synthesis of acid and neutral proteases. However, we assume that coordination complex may not induce changes in the structure of the proteases themselves. Some of our studies of the mechanism of the influence of the metal complexes on the exocellular hydrolases by micromycetes showed that coordination compounds promote the synthesis of new proteins, but did not affect the structure of the enzymes. Thus, the SDS-PAGE of the α-amylase from *Aspergillus niger* 33-19 CNMN FD 02A strain cultivated with supplementation of 10 mg/L $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound in the nutrient medium and purified through ion exchange chromatography demonstrated similar results obtained for the micromycete grown in standard conditions, without coordination complex [6, 7].

Although, other our researches on stimulatory effect of the coordination compounds of transition metals showed that the metal complexes rather influence the biosynthesis of the enzymes than activate the already synthesized fungi exoenzymes due to the inclusion in enzyme structure. The direct contact between some metal complexes of cobalt and copper with oxime ligands and exoprotease containing cultural liquid of *Fusarium gibbosum* CNMN FD 12 strain presented an inhibitory effect on neutral proteases [8].

Coordination compounds of metals are known to be more stable, less toxic and in consequence more active as their inorganic salts. Entering into the cell they initiate a simple chemical reaction, followed by a cascade reaction of biomolecules complex, finally resulted in a broad physiological effect on the whole organism [5]. From the foregoing, it is apparent that studies on the biological properties of the coordination compounds are very promising as a theoretical and a practical point of view. Additional challenge remains the improvement of the methods for detection of their biological activity.

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ANNEX

Synthesis of the [Cu(H_2L)Cl] complex and physical measurements.

The synthesis of the [Cu(H_2L)Cl] (**1**) compound and the corresponding ligand was performed using as main chemical reagents: thiosemicarbazide, copper (II) chloride dihydrate, 4-formyl-3-hydroxy-2-naphthoic acid.

The proposed for biological studies complex **1** was synthesized in two steps. Initially, a new ligand was synthesized by the condensation reaction of 4-formyl-3-hydroxy-2-naphthoic acid with the thiosemicarbazide (CH_5N_3S) in the 1:1 molar ratio. New thiosemicarbazone ligand H_3Nthios·CH_3OH ($C_{13}H_{11}N_3SO_3 \cdot CH_3OH$), named 4-[(ε)-(carbamothioilhidrazono)]-3-hidroxinafthalene-2-carboxylic acid methanol solvate was synthesized in methanol solution, and separated as yellow-orange crystals. On the data of elemental analysis (in %): founded experimental - C 52.21; H 4.53; N 13.08; calculated for the formula $C_{14}H_{15}N_3SO_4$ - C 52.33; H 4.67; N 13.15 and NMR spectroscopy spectra measured on Bruker AV600 spectrometer (**^{13}C NMR** (600 MHz, *(CD3)2SO*), δ (ppm): 177.8, 171.8, 157.8, 141.3, 135.2, 133.6, 131.1, 130.5, 126.8, 125.0, 124.5, 115.0, 112.7; ***^{1}H NMR*** (600 MHz, *(CD3)2SO*) δ (ppm): 11.53 (s, 1 H), 8.91 (d, *J* = 8.4 Hz, 1 H), 8.86 (s, 1 H), 8.60 (s, 1 H), 8.18 (s, 1 H), 8.02 (d, *J* = 7.8 Hz, 1 H), 7.67 (m, 1 H), 7.59 (s, 1 H), 7.43 (m, 1 H); the structure and composition of ligand· was revealed. The ligand was synthesized with 60% of yield. The melting point of the ligand constitutes 228°C. IR (ATR mode), cm^{-1}: 3451, 3419, 3276, 3147,3013, 2977, 2814, 2569, 1738, 1694, 1674, 1623, 1603, 1578, 1545, 1455, 1433, 1406, 1392, 1372, 1350, 1294, 1279, 1241, 1211, 1173, 1159, 1121, 1068, 1035, 998, 957, 937, 910, 866, 835, 793, 744, 733, 723, 685, 665, 653, 610, 597, 545, 477, 442, 424.

In continuation, the ligand H_3Nthios·CH_3OH was involved in the reaction with copper(II) chloride salt. Reaction of ethanolic solution of the ligand and copper(II) chloride salt the titled thiosemicarbazone coordination compound of formulae [Cu(H_2L)(Cl)] (**1**) was separated. The detailed procedure of synthesis constitutes in followings: 0,34 g (0,002 mol) of $CuCl_2 \cdot 2H_2O$ were dissolved in 20 mL of ethanol and added to 0,64 g (0,001mol) of $H_3L \cdot CH_3OH$ ligand **1** dissolved separately in 50 mL tetrahydrofuran, with stirring. Green sediment is immediately formed after 0.5 h of stirring. The formed sediment was filtered, washed with ethylic alcohol and dried under air. The yield of compound is 80 %. From the founded elemental analysis data (in %): (C 40.19; H 2.41; N 10.57, Cl 8.76) in comparison to theoretic calculated: C 40.32, H 2.58, N 10.85, Cl 9.16, the composition of the complex [Cu(H_2L)(Cl)] (**1**) was established. The absorption bands in the IR spectrum of the complex are, (cm^{-1}): 3419, 3276, 3218, 3179, 3058, / 1678, 1614, 1593, 1579, 1545, 1508, 1457, 1429, 1405, 1377, 1360, 1338, 1230, 1215, 1201, 1173, 1114, 998, 968, 937, 910, 866, 796, 763, 736, 704, 678, 624, 610, 578, 542, 521, 482, 434, 428.

The complex **1** are soluble in ethyl alcohol, DMSO, DMF, less soluble in chloroform, acetone and is insoluble in water. The parameters of EPR spectra recorded on microcrystalline sample at room temperature on AVANCE 400 spectrometer ($g_\perp = 2.039$, $g_{II} = 2.165$ $g_0^* = 2.053$) suggest the presence of copper(II) ions and a square-planar structure of the complex. Based on the presented data, was supposed that the ligand is in monodeprotonated form H_2L^- and chelates to copper(II) ion in thiolate form by ONS set of donor atoms, the fourth position being occupied by Cl^- anion.

Elemental analyses (C, H, N,) were performed on a Elemental Analyzer vario EL(III). IR spectra were recorded on a Perkin Elmer spectrum 100 FT IR Spectrometer in the 4000-600 cm^{-1} range [1].

[1] Nakamoto, K., (1970): Infrared spectra of inorganic and coordination compounds. Second Edition. John Wiley and Sons, New York, 338 p.

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