# THE EFFECT OF SOME METAL COMPLEXES OF OXIME LIGANDS ON PROTEOLYTIC ACTIVITY OF *Fusarium gibbosum* CNMN FD 12 STRAIN

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**Abstract.** The supplementation of Co(III) and Cu(II) coordination compounds to the nutrient medium of *Fusarium gibbosum* CNMN FD 12 essentially increases fungal proteolytic activity. The maximal stimulatory effect was obtained on the 5th day of cultivation. Depending on type and concentration, the compounds stimulated the activity of neutral proteases with 17.5-96.0% and the activity of acid proteases with 12.1-71.7%. On the 6th day of cultivation fungal proteolytic activity decreased significantly, in most cases below the control sample. The direct supplementation of coordination compounds to cultural liquid containing enzyme indicated a neutral or inhibitory effect of just 21.2% compared to the control. This demonstrated the indirect stimulatory effect of coordination compounds on proteolytic activity, most likely due to the inclusion of them in cell metabolism and not direct - in enzyme structure. The chelating reaction with EDTA showed 81.8-78.8% remaining activity of neutral proteases at concentration of 1-10mM EDTA. This result allows us to suppose that most *F. gibbosum* exoproteases are not metalloproteases. They can belong to family of extracellular serine proteases and aspartic proteases.

**Keywords***: Fusarium gibbosum* (*Gibberella intricans*), neutral and acid protease, coordination compounds, oxime ligands

## INTRODUCTION

Statistically, proteases represent one of the three largest groups of industrial enzymes and account for 60% of the total worldwide sale of enzymes, about US$ 2.0 billion [18]. Proteases are a complex group of enzymes, varied by substrate specificity, reaction mechanism, stability to pH and temperature. Due to the specificity of action and variety of properties, proteases have numerous applications in economy, mainly in the food, leather, pharmaceutical industries, diagnostics and zootechny. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in production of biodegradable detergents and several bioremediation processes [3, 17, 21].

Microscopic fungi are advantageous sources of proteases, having the particularity to grow on cheap media prepared from food processing by-products. Micromycetes produce significant amounts of exocellular proteases easily recoverable from culture medium by simple and accessible techniques, have adaptive metabolism and wide range of proteolytic enzymes, active in a large pH range (4-11) [4, 22, 27].

The biotechnological production of enzyme preparations involves maximal exploitation of microorganisms' biosynthetic potential. Previous studies to increase biosynthesis of fungi exocellular hydrolases (lipases, cellulases, amylases, pectinases) have revealed stimulatory effect of some metal complexes on growth and enzyme biosynthesis of mycelial fungi, reducing the technological cycle and directing differentially obtaining of enzyme products [5, 24].

Thus, the addition of $[Co(Niox)_2(An)_2][BF_4]$ coordination compound to growth medium of *Aspergillus niger* CNMN FD 01 – producer of lipases, increased the enzyme activity of strain over 2 times [28]. Coordination compounds of cobalt $[Co(DH)_2(Thio)_2]_3F[SiF_6]\cdot 1{,}5H_2O$, $[Co(DH)_2(Thio)_2]_2[SiF_6]\cdot 3H_2O$ and $[Co(DH)_2(Thio)_2][BF_4]\cdot 3H_2O$ showed stimulator effect of 1.9-2.3 times on *Rhizopus arrhizus* F 67 pectinases biosynthesis [7]. Metal complexes $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$, $[Co(NioxH)_2(Sam)_2]_2[TiF_6]\cdot 3H_2O$ and $[Co(NioxH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ in concentrations of 5-10mg/L increased amylolytic activity of *A. niger* 33-19 CNMN FD 02A with 23-64% and reduced growth cycle by 24-48 hours [29].

It is well known that most proteases are metalloenzymes and requires metal ions for molecular structure stability and catalytic activity. Almost all proteases contain zinc ions in the active site, but several enzymes contain one or two cobalt or manganese ions [10]. A cobalt-dependent amino-peptidases from *Escherichia coli* [25] and *Saccharomyces cerevisiae* [13], and a manganese-dependent alkaline serine protease produced by some *Bacillus* strains were detected [14]. Also, a series of studies have shown that zinc ions from active site of naturally zinc metalloproteases could be substituted by other metal ions (cobalt, manganese, copper, nickel), without affecting or sometimes activating catalytic properties of the enzyme [16, 20]. The alkaline proteases from *Bacillus licheniformis* require calcium, manganese or magnesium ions to stabilize enzyme activity at high temperature [1]. The proteolytic activity of *Bacillus* increased in presence of $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Cu^{2+}$ and $Hg^{2+}$ ions [26]. Therefore, most fermentation mediums require divalent metal ions for optimum production of proteases by microorganisms [15].

The aim of the research was to evaluate the effect of some metal complexes of cobalt and copper with oxime ligands on proteolytic activity of *Fusarium gibbosum* fungal strain - producer of acid and neutral proteases.

## MATERIALS AND METHODS

**Microorganism and culture conditions:**

*Fusarium gibbosum* (*Gibberella intricans*) CNMN FD 12 fungal strain was isolated from infected plants of winter wheat and is stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova [8]. The influence of following coordination compounds was tested: $[Co(DH)_2(An)_2][PF_6]$ (**1**), $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ **(2),** $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ (**3**), $[Cu(DSamH_2)_3]SO_4\cdot 5H_2O$ (**4**) (DH - monoanion of dimethylglyoxime, An - aniline, NioxH - monoanion 1.2-cyclohexanedione dioxime, Thio - thiocarbamide, DMF - dimethylformamide, Seu - selenocarbamide, Se-Seu - seleno-selenocarbamide, $DSamH_2$ - disulfanilamide glyoxime).

Submerged fermentation of *F. gibbosum* was performed in Erlenmeyer flasks of 500ml volume, at 28-30°C, on shakers (180-200rpm), during 4-6 days. Each flask contained 100ml of nutrient medium of the following optimal composition (g/L): corn flour - 20.0, soybean flour - 10.0, $CaCO_3$ - 2.0; $(NH_4)_2SO_4$ - 1.0. Nutrient medium pH was 6.25. Coordination compounds were added in form of solution of 0.1% after autoclavation. The concentration of compounds in medium was 1.0, 5.0, 10.0 and 15.0 mg/L. Variants cultivated without metal complexes served as control.

The solution of spore suspension with density $3x10^6$ spores/ml, obtained by washing with sterile water the 15 days culture grown on malt-agar oblique columns, was used as seed material. The concentration of inoculum to each flask was 10% v/v [2].

**Enzyme assay:**

Proteolytic activity was determined in the cultural liquid after 4-6 days of *F. gibossum* cultivation. The medium was separated from biomass by filtration. Activity of exocellular protease was determined according to the Willstatter method by measuring the amount of released free carboxyl groups in ethanol solution of amino acids and polypeptides obtained at hydrolysis of 5% gelatine substrate with pH 7.4 for neutral proteases and pH 3.6 for acid proteases, after 3 hours incubation at 40°C. The reaction mixture contained 10ml of buffered (0.2M phosphate buffer adjusted to pH 7.4 or 3.6 with 0.1M citrate buffers) 5% gelatine solution and 2ml fungal cultural liquid. The reaction was stopped and developed with 1% thymolphthalein solution in 96% ethanol in ratio: 1ml reaction mixture/20ml ethanol. The samples were titrated against 0.1N NaOH until permanent blue colour. The immediately stopped and titrated reaction mixture, without incubation, served as control [11].

**Determination of direct effect of metal complexes:**

Four reaction solutions with concentration of 5mg/L coordination compounds each from 5 day cultivated nutrient medium were prepared. After 1 hour incubation at 5°C, the activity of neutral proteases (%) was assayed.

**Protease inhibitors assay:**

The effect of protease inhibitor ethylenediaminetetraacetic acid (EDTA) was tested. The control (without coordination compounds) cultural liquid was maintained with 1, 5, 10 mM EDTA solution for one hour at 5°C. The remaining protease activity (%) was measured by standard neutral protease determination.

**Statistical analysis:**

All the experiments performed three times and the results are presented as the average of three with standard deviation of the average. The level of significance p is $\leq 0.05$ [9].

## RESULTS

The influence of coordination compounds of Co(III) and Cu(II) with oxime ligands on proteolytic activity of *Fusarium gibbosum* CNMN FD 12 fungal strain was evaluated in dynamics, during the 4-6 days of cultivation. Earlier studies showed the maximal exoproteases biosynthesis by *F. gibbosum* in the 5-6 days grown culture [8].

It was observed that all studied metal complexes had a stimulator effect on proteolytic activity of micromycete *Fusarium gibbosum*, the increase in activity depending on the compound concentration (Tables 1 and 2). The peak of acid and neutral protease activity was determined on the 5th day of cultivation, similar to the control sample. On the 6th day of cultivation fungal proteolytic activity decreased significantly, in most cases below the control.

The $[Co(DH)_2(An)_2][PF_6]$ addition to nutrient medium of *F. gibossum*, presented an increase of neutral protease activity of 17.5-92.5% compared to the control sample (Figure 1A). The maximal proteolytic activity of 6.468U/ml was observed for 10mg/L coordination compound (Table 1). Significant is that activity of neutral protease in the 4th day of culture growth with 5mg/L compound was 10% higher compared to enzyme activity of control sample on the 5th day of cultivation. The $[Co(DH)_2(An)_2][PF_6]$ increased activity of acid proteases with 12.1-48.5% (Figure 1B). The maximal proteolytic activity of 4.116U/ml was observed for 10mg/L compound (Table 2).

In the case of $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ compound, neutral protease activity on the 5th day was 5.376-6.300 U/ml (Table 1), the increase representing 50-87.5% (Figure 1A). Acid proteases activity varied between 3.192 and 4.530 U/ml (Table 2), higher with 15.2-63.6% compare to the control (Figure 1B). The maximal activity of both proteases was observed at concentration of 5mg/L. Similar to previous compound **1**, $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ accelerated the increase of proteolytic activity. On the 4th day of cultivation enzyme activity of neutral and acid proteases in coordination compound containing samples was practically equivalent with control on the 5 day of growth. For neutral proteases this was observed at concentration of 5mg/L and for acid proteases – at 15mg/L.

**Table 1.** The effect of coordination compounds with oxime ligands on activity of neutral protease from *Fusarium gibbosum* CNMN FD 12

| Coordination compounds | Concentration, mg/L | Activity of neutral protease, U/ml | | |
|---|---|---|---|---|
| | | 4th day | 5th day | 6th day |
| $[Co(DH)_2(An)_2][PF_6]$ | 1 | 3.570±0.04 | 4.788±0.08 | 2.016±0.05 |
| | 5 | 3.696±0.07 | 5.208±0.07 | 2.352±0.04 |
| | **10** | 2.772±0.04 | **6.468±0.04** | 0.504±0.05 |
| | 15 | 2.856±0.07 | 3.948±0.07 | 0.392±0.02 |
| $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ | 1 | 2.334±0.11 | 4.850±0.04 | 2.039±0.05 |
| | **5** | 3.612±0.04 | **6.300±0.04** | 2.520±0.07 |
| | 10 | 3.024±0.07 | 5.040±0.08 | 3.864±0.04 |
| | 15 | 3.108±0.01 | 5.376±0.08 | 1.932±0.07 |
| $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ | 1 | 1.934±0.09 | 6.342±0.11 | 1.490±0.05 |
| | **5** | 3.612±0.04 | **6.048±0.07** | 1.260±0.01 |
| | 10 | 1.680±0.01 | 5.712±0.07 | 1.260±0.04 |
| | 15 | 1.008±0.04 | 4.704±0.04 | 1.050±0.04 |
| $[Cu(DSamH_2)_3]SO_4\cdot 5H_2O$ | **1** | **2.267±0.08** | **6.586±0.05** | 2.431±0.08 |
| | 5 | 2.016±0.04 | 6.300±0.04 | 3.528±0.04 |
| | 10 | 1.596±0.04 | 5.964±0.07 | 4.200±0.08 |
| | 15 | 1.344±0.07 | 4.788±0.04 | 2.520±0.07 |
| Control | 0 | 1.260±0.01 | 3.360±0.07 | 2.520±0.04 |

**Table 2.** The effect of coordination compounds with oxime ligands on activity of acid protease from *Fusarium gibbosum* CNMN FD 12

| Coordination compounds | Concentration, mg/L | Activity of acid protease, U/ml | | |
|---|---|---|---|---|
| | | 4th day | 5th day | 6th day |
| $[Co(DH)_2(An)_2][PF_6]$ | 1 | 0.346±0.11 | 3.124±0.04 | 0.414±0.02 |
| | 5 | 0.336±0.01 | 3.150±0.04 | 0.420±0.04 |
| | **10** | 0.420±0.04 | **4.116±0.04** | 0.504±0.04 |
| | 15 | 0.756±0.07 | 3.108±0.04 | 0.392±0.02 |
| $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ | 1 | 0.714±0.08 | 3.276±0.11 | 1.267±0.04 |
| | **5** | 0.756±0.04 | **4.530±0.03** | 1.596±0.04 |
| | 10 | 1.512±0.01 | 3.528±0.04 | 1.512±0.04 |
| | 15 | 2.688±0.04 | 3.192±0.07 | 0.420±0.04 |
| $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ | 1 | 0.630±0.04 | 3.280±0.04 | 1.050±0.08 |
| | 5 | 0.168±0.01 | 4.284±0.07 | 0.672±0.01 |
| | **10** | 0.756±0.07 | **4.704±0.04** | 0.420±0.01 |
| | 15 | 0.084±0.01 | 4.536±0.07 | 0.588±0.04 |
| $[Cu(DSamH_2)_3]SO_4\cdot 5H_2O$ | **1** | 0.798±0.04 | **4.759±0.08** | 2.290±0.01 |
| | 5 | 0.252±0.01 | 4.284±0.07 | 2.016±0.04 |
| | 10 | 0.588±0.04 | 2.688±0.07 | 0.756±0.07 |
| | 15 | 0.420±0.01 | 2.502±0.07 | 0.168±0.01 |
| Control | 0 | 0.504±0.04 | 2.772±0.04 | 1.176±0.07 |

The effect of metal complex $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ on proteolytic activity of *F. gibossum* was identical to first two compounds of Co(III) described. The highest enzyme activity from the 5th day was for 5mg/L and 10mg/L compound, showing an increase of neutral protease activity by 80% and acid protease activity by 69.7% (Figure 1). On the 4th day of culture growth, in concentration of 5mg/L of compound the activity of neutral proteases is comparable with control sample from 5th day, the level of acid proteases remaining low (Table 1 and 2).

The coordination compound of Cu(II) enhanced both proteolytic activities at minimal tested concentration of 1mg/L, the enhancing effect is visibly reduced with the concentration increase. The activity of neutral and acid proteases on 5th day of cultivation was 96.0% and, respectively, 71.7% higher compared to control sample from the same day (Figure 1).

The stimulatory effect of coordination compounds of Co(III) and Cu(II) with oxime ligands on proteolytic activity of *Fusarium gibbosum* CNMN FD 12 is obvious. To establish that coordination compounds exercise direct action on proteolytic enzymes released onto the cell or have stimulatory effect on protease biosynthesis by *F. gibbosum*, solutions of cultural liquid of 5 days with 5mg/L compound were prepared and the remaining activity of neutral proteases was evaluated (Figure 2). The results demonstrated that studied metal complexes did not have direct stimulatory effect on the activity of neutral proteases. The compounds **1** and **2** showed the same values as the control sample and the compounds **3** and **4** decreased proteolytic activity by 21.2% after 1 hour incubation of 5mg/L compound.

To partially estimate the nature of exoproteases from nutrient medium of *F. gibbosum* CNMN FD 12

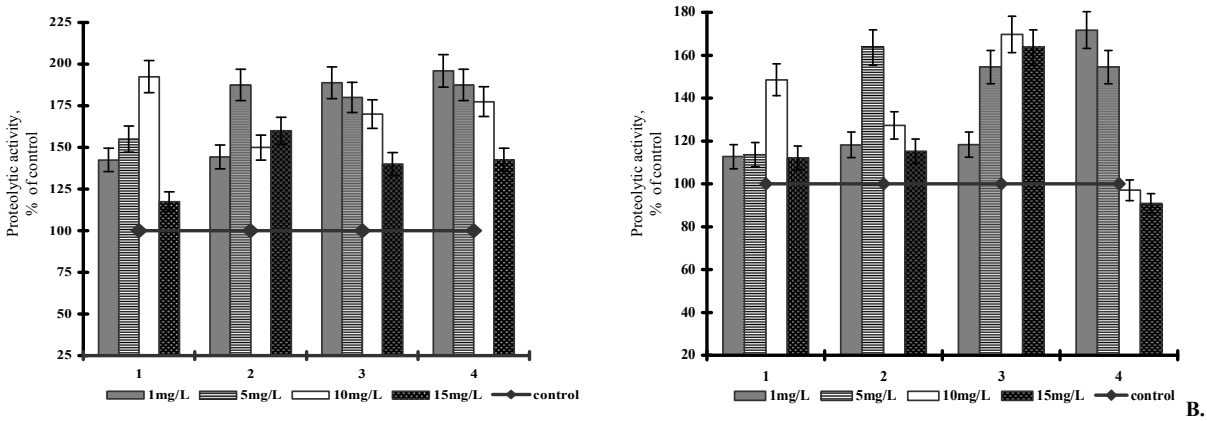


**Figure 1.** The effect of coordination compounds with oxime ligands $[Co(DH)_2(An)_2][PF_6]$ (**1**), $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ (**2**), $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ (**3**), $[Cu(DSamH_2)_3]SO_4\cdot 5H_2O$ (**4**) on activity of neutral (A) and acid proteases (B) on the 5 day of *Fusarium gibbosum* cultivation

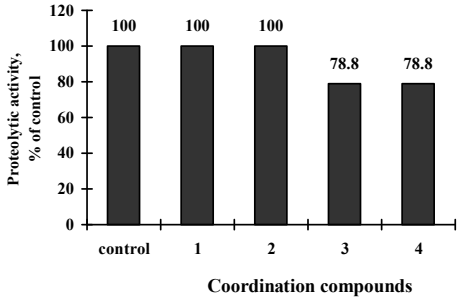


**Figure 2.** The direct influence of coordination compounds with oxime ligands $[Co(DH)_2(An)_2][PF_6]$ (**1**), $[Co(NioxH)_2(Thio)_2][PF_6]\cdot 0.5DMF\cdot 0.5H_2O$ (**2**), $[Co(DH)_2(Seu)_{7/4}(Se\text{-}Seu)_{1/4}]_2[TiF_6]\cdot H_2O$ (**3**), $[Cu(DSamH_2)_3]SO_4\cdot 5H_2O$ (**4**) on activity of neutral proteases of *Fusarium gibbosum* CNMN FD 12

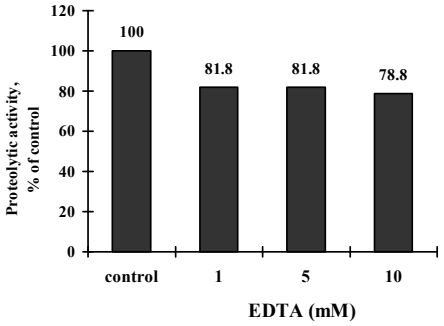


**Figure 3.** The influence of EDTA on activity of neutral proteases of *Fusarium gibbosum* CNMN FD 12

the reaction with EDTA – chelating agent of metalloproteases was performed (Figure 3).

The study indicated the low inhibition of neutral proteases activity at standard inhibitory concentration (1-10mM) of EDTA. The proteolytic activity decreased by 18.2% at the concentrations 1-5mM and by 21.2% at 10mM EDTA.

## DISCUSSIONS

The importance of coordination compounds for biotechnology is recognized and biological effect of some compounds is intensely investigated in the recent years [6, 24, 30].

The studied metal complexes of cobalt and copper with oxime ligands confirmed earlier researches and demonstrated visible positive effect on enzyme activity of neutral and acid proteases by *Fusarium gibbosum* CNMN FD 12. The stimulatory effect on 5th day of micromycete cultivation varied within 17.5-96.0% for neutral proteases and 12.1-71.7% for acid proteases, depending on type and concentration of compound. Also, the metal complexes **1-3** accelerated the synthesis of neutral (compounds **1** and **3**) or both neutral and acid (compound **2**) proteases with 24 hours, indicating on the 4th day of culture growth same values or higher as the value of control sample on the 5th day of cultivation. This fact has importance especially for enzyme biotechnology and can be further successfully developed.

As for the mechanism of coordination compounds influence on biosynthesis of fungi is poorly elucidated, although a variable group of microorganisms and compounds was investigated. It was observed that the biological effect varies by different type of coordination compounds and must be investigated for each group of microorganisms. It is proposed that microelements from structure of coordination compounds participate directly or indirectly in cell metabolism, intensifying synthesis of bioactive substances, including enzymes. On the other hand microelements can act directly on already synthesized enzymes, activating, stabilizing or inhibiting them [12].

The investigation of direct influence of metal complexes **1-4** on proteolytic activity of *F. gibbosum* CNMN FD 12 demonstrated that on direct contact with enzymes these compounds inhibit the activity of neutral proteases. Thus, it can be affirmed that stimulatory effect of studied coordination compounds was due to the intensification of proteases biosynthesis by micromycete *F. gibbosum* and not due to the direct inclusion in enzyme structure.

Coordination compounds of metals, known to be more stable, less toxic and in consequence more active as their inorganic salts are an important source of elements for cell metabolism. 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 [19].

The exocellular proteases of fungi are represented by a complex of proteases, most frequently reported being metalloproteases (metalloexopeptidases) [23]. Metalloproteases are activated by metal ions and are completely inactivated by chelating agents, such as EDTA. The low inhibition range (18-21%) of 1-10mM EDTA solution on neutral proteases activity indicates that most *F. gibbosum* CNMN FD 12 proteases are not metalloproteases. They can belong to family of extracellular serine proteases and aspartic proteases [31].

To conclude, the tested metal complexes visibly intensified the proteolytic activity of neutral and acid proteases by *Fusarium gibbosum* CNMN FD 12 strain and can be recommended as regulators of fungal synthesis.

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