THE EFFECT OF $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ COORDINATION COMPOUND ON POLYPEPTIDE SPECTRUM OF AMYLOLYTIC PREPARATION FROM *Aspergillus niger* 33-19 CNMN FD 02A STRAIN

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Abstract. From mycelial fungus *Aspergillus niger* 33-19 CNMN FD 02A cultivated on nutrient medium supplemented with $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound amylolytic preparation with total α-amylase activity of 40042U/g and a specific activity of 131.3U/mg proteins was obtained. Through gel filtration and ion exchange chromatography enzyme preparation was purified 7.8 fold with a yield of 13%. SDS-PAGE electrophoresis of purified α-amylase revealed 2 polypeptide bands of 66 and 40.5kDa identified as α-amylase isomorphs similar to previously obtained results on *Aspergillus niger* grown without any coordination compounds. $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ increase the activity of non-purified amylolytic preparation, most likely due to the bonds formed between metal complex and some α-amylase radicals.

Keywords: *Aspergillus niger*, α-amylase, enzyme purification, coordination compounds, SDS-PAGE

INTRODUCTION

Until now, major industrial applications of *Aspergillus* species remain associated with traditional food and fodder production by fermentation [25]. Industrial obtaining of microbial enzymes is economically profitable at the selection of strains with high biosynthetic activity, stable over time, and at application of effective methods for adjusting the biosynthetic potential of strain-producers, that would allow assigning the superior qualitative properties to obtained enzyme complex and, thus, extending the fungal enzymes application fields [1, 8]. Numerous studies in biotechnology have demonstrated the possibility of applying coordination compounds of transition metals as stimulators and regulators of microbial synthesis [7, 11].

Increased interest in metal complexes is induced by their physiological properties. Biological characteristics of coordination compounds are largely attributed to the presence in their composition of metal atoms: Co, Cu, Fe, Mn, Zn, Ni, Mo etc. Ordinary, microelements represent an extremely small quantity of the cells. The major part of microelements forms the enzyme systems of organisms, determining their functionality [19, 20].

The high activity of metal complexes is explained by the energy and conformation state of the molecules, conditioned by spatial geometry, unusual system of the length bonds, coordination number etc. [12, 21]. The elements bonded to bioligands are less toxic and more catalytic active compared to their inorganic forms. This contributes significantly to the synthesis of a wide variety of coordination compounds and to expanding the areas of microelements application. Due to the physiological activity of coordination compounds, low toxicity, positive effects on biosynthetic processes of microorganisms, reported in the literature, the use of coordination compounds as enhancer of biosynthetic capacities of fungal cells, without genetic modification, offers opportunities [5, 22].

The feature of metal ion to fulfil its role in the active center of the enzyme depends on the property of the metal ion to generate complexes, geometry and stability of these complexes [13]. Thus, special attention is given by researchers to the ability of transition metals to form complexes with oximes, not only for the B_{12} or haemoglobin models synthesis, but as substances that exhibit a wide range of synthetic, analytical and structural possibilities as well. Inclusion of fluoride ions or fluorine anions in cobalt dioximes allowed assembling of new different complex molecules against other halogens [6, 21].

Thus, the introduction in the nutritive medium of *A. niger* 412 - lipases producer of metal complexes of cobalt (III) with monoximes (MH) - $[Co(MH)_2(Py)_2][BF_4]\cdot H_2O$ increased enzymatic activity of the strain with 30.0 to 46.2% [10]. For *A. niger* 33 CNMN FD 06A strain - amylases producer the stimulator effect of coordination compounds of cobalt (III) with dioximes (DH) $[Co(DH)_2(Thio)_2]_3F[SiF_6]\cdot 1.5H_2O$, $[Co(DH)_2(Thio)_2]_2[SiF_6]\cdot 3H_2O$, $[Co(DH)_2(Thio)_2][BF_4]\cdot 3H_2O$ was observed, which increased enzyme biosynthesis of the strain with 26.3-42.6 % [9].

Amylolytic activity is strongly modified by the presence of the compound $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$, which contains titanium (Ti^{4+}) metal in the external field, in composition of fluorinated anion. At addition to the nutrient medium of *A. niger* 02A 33-19 FD CNMN strain of $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound in concentration of 10.0 mg/L, amylase biosynthesis increased with 23.7-28.9% compared to the control and the life cycle reduced by 24 hours [21, 23].

The stimulating effect is also confirmed by microbiological research which shows the earlier

appearance of abundance of well developed mycelium and active sporulation.

The aim of the research was to evaluate the effect of $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound on amylolytic activity and polypeptide spectrum of enzyme preparation obtained from cultural liquid of micromycete *A. niger* CNMN 33-19 FD 02A, submerse cultivated in the presence of this metal complex.

MATERIALS AND METHODS

Microorganism and culture conditions:

Object of study, strain of mycelial fungi *Aspergillus niger* 33-19 CNMN FD 02A – producer of amylases, stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova, was cultivated in Erlenmeyer flasks of 1000ml volume, at temperature of 28-30°C, on shakers (180-200rpm), during 5 days. Each flask contains 200ml of nutrient medium of the following, chosen as optimal, composition (g/L): starch - 3.0; bean flour - 9.0; wheat bran - 18.0; KH_2PO_4 - 2.0; KCl - 0.5; $MgSO_4$ - 0.5 and $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ in concentration of 10.0 mg/L. Coordination compound was added to the autoclaved cultivation medium in the form of solution, prepared at dissolving 100mg of the compound in 100mL sterile distilled water. Initial nutrient medium pH was 3.0 [9]. As control served variants of *Aspergillus niger* 33-19 CNMN FD 02A, grown standard, without metal complexes.

Spore suspension in quantity of 10% v/v with density $3\cdot 10^6$ spores/ml, obtained by washing with sterile water a 12-14 days culture grown on malt-agar oblique columns was used as seed material [2].

Enzyme extraction:

After 5 days of cultivation the medium was separated from biomass by filtration and centrifuged 20 minutes at 4000-6000rpm. The α-amylase was isolated from supernatant with 96% ethanol cooled to -15°C in ratio 1:4 C_2H_5OH, sedimentation duration - one hour, concentration of $CaCl_2$ – 0.2%. The sediment was centrifuged 20 minutes at 6000rpm and dried at 20-22°C [23]. Obtained enzyme preparation was further investigated and purified.

Enzyme assay:

α-Amylase activity in enzyme preparation was determined according to the modified SKB method [14]. One unit of activity is defined as the amount of enzyme, which catalyzes hydrolysis of one gram soluble starch up to dextrins with different molecular weight; hydrolysis range 30% under the assay conditions. 10ml of buffered (0.2M acetate buffer, pH 4.7) 1% soluble starch (Sigma) solution as the substrate and 5ml adequately diluted enzyme sample (5ml distilled H_2O for control) was incubated 10 minutes at 30°C. The reaction was stopped and developed with iodine solution in ratio 0.5ml reaction mixture/50ml iodine solution (5mg iodine and 50mg KI in 100ml 0.1N HCl) for 5 minutes. Absorbance of control and experiment samples were measured at 656nm.

Protein assay:

Total protein content was determined according to the Lowry method of taking crystalline bovine serum albumin as the standard [16].

α-Amylase purification:

Gel filtration. 75mg dried enzyme preparation was washed with 3ml 20mM Tris-HCl buffer (pH 7.0), centrifuged for 10min at 14000rpm. 2.5ml supernatant was applied to PD-10 column (Amersham Pharmacia Biotech) equilibrated with 20mM TRIS-HCl buffer, pH 7.0. The column was eluted with same buffer.

Ion exchange chromatography. Polled fraction was loaded onto an anion exchange HiTrap™ Q (5ml) column at flow rate 1ml/min FPLC System (Amersham Pharmacia). The column was equilibrated with 20mM Tris-HCl buffer. Unbound proteins were eluted with linear gradient of 0-0.5M NaCl in the same buffer. In each eluted fraction amylolytic activity and protein was assayed.

Gel electrophoresis:

Isolated proteins from amylolytic active fractions and from initial amylolytic preparation were applied to 15% SDS-PAGE electrophoresis, according to the Laemmli method, to evaluate purity of α-amylase [15]. Gel was stained with coomasie Brillant Blue R-250. Molecular weight markers were β-Galactosidase (116kDa), Phosphorylase b (97.4kDa), Bovine serum albumin (66.2kDa), Alcohol dehydrogenase (37.6kDa), Carbonic anhydrase (28.5kDa).

Statistical analysis:

All the experiments performed thrice and the results are presented as the mean of three, with the level of significance $p \leq 0.05$ [17].

RESULTS

Cultivation of micromycete *A. niger* 33-19 CNMN FD 02A on nutrient medium supplemented with 10mg/L $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound lead to obtaining of a new enzyme preparation, with an amylolytic activity of 40042U/g and a specific activity of 131.3U/mg proteins (Table 1). Compared with previous described results, α-amylase activity of dried amylolytic preparation increases with 23% under the influence of studied metal complex. However, the specific activity of new preparation is practically identical, presenting a minimum difference of 5.3% advantage for enzyme preparation produced traditionally, without coordination compound. This is due to 30% enhancement of total protein amount from amylolytic preparation, obtained with $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ application as stimulator and regulator of fungi synthesis.

Further purification of amylolytic preparation (I), obtained from cultural liquid of micromycete *A. niger* CNMN 33-19 FD 02A, submerse cultivated in presence of $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ metal complex, by gel filtration and ion exchange chromatography indicate the last increase of α-amylase

specific activity to 1019.30U/mg protein with a purification fold of 7.8 times (Table 2). The yield of α-amylase was 13%.

Similar to previous data, from all separated fractions of protein by ion exchange chromatography (Fig. 1), only a few fractions contain α-amylase, with a peak of amylolytic activity in 2 fractions, numbers 14 and 15, and a little difference of 4.09U/ml, or 7% between them. The peak of eluted proteins resulted in fraction 14, presenting an amount of 0.098mg/ml proteins. Difference in α-amylase specific activity shows a 25% advantage for fraction 15, with a value of 1158.69U/ml protein. For presentation of ion exchange chromatography step from purification scheme (Table 2), both high active fractions 14 and 15 were combined and recalculated their 1.5 times dilution, used for solution loaded onto ion exchange column.

Evaluation of purity of amylolytic preparation and amylolytic active fraction eluted after ion exchange chromatography confirms the heterogeneous protein content of initial preparation, expressed in a totality of polypeptide bands with different molecular weights (Fig. 2). Appeared polypeptide bands show little difference between proteins of amylolytic preparation obtained under influence of $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ and in the absence of them [3].

SDS-PAGE of solution obtained after ion exchange chromatography shows purification of α-amylase. Two polypeptide bands with apparent molecular masses of 66kDa and 40.5kDa were observed. The appeared polypeptide bands were identified as 2 isomorphs of α-amylase. The data is similar with results obtained at purification of amylolytic preparation from *A. niger* 33-19 CNMN FD 02A strain cultivated without coordination compounds [3] and demonstrate that $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ not induces changes in structure or synthesis of extracellular α-amylase.

Table 1. Amylolytic properties of enzyme preparations obtained from micromycete *A. niger* 33-19 CNMN ED 02A

Variants	Protein (%)	α-Amylase activity (U/g)	Specific activity (U/mg)
A. niger 33-19 CNMN ED 02A grown with 10mg/L $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ supplementation **(I)**	30.5	40042.0	131.3
A. niger 33-19 CNMN ED 02A grown in standard conditions [3] **(II)**	23.5	32554.5	138.3

Table 2. Purification profile of α-amylase from amylolytic preparation (I)

Purification Steps	Volume (ml)	α-Amylase activity (U/ml)	Total α-Amylase activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Amylolytic preparation solution (25mg/ml)	3	1001.05	3003.15	7.625	22.875	131.28	100	1
Extract	3	627.78	1883.37	2.340	7.020	268.28	63	2.0
PD-10 column gel filtration	3	535.68	1607.04	1.656	4.968	323.48	54	2.5
HiTrap™ Q column ion exchange chromatography	3	131.49	394.47	0.129	0.387	1019.30	13	7.8

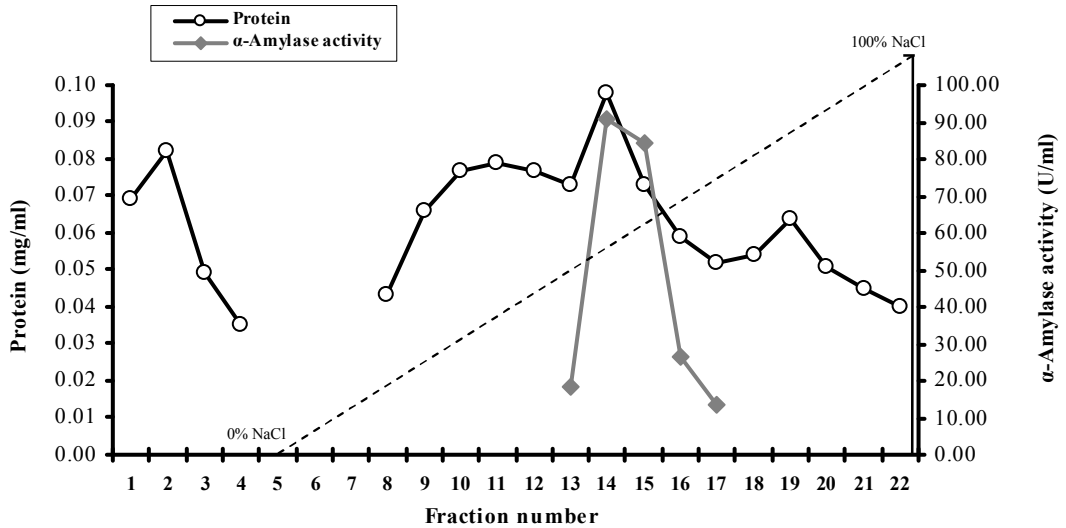

Figure 1. Purification of α-amylase from amylolytic preparation (I) by ion exchange chromatography. Sample: 2ml of α-amylase preparation after gel filtration through PD-10 column. Column: HiTrap™ Q (5ml). Flow rate: 1ml/min. Buffer A: 20mM Tris-HCl, pH 7.0. Buffer B: A+0.5M NaCl. Gradient: 0% B for 27ml, 0%-100% B in 75ml

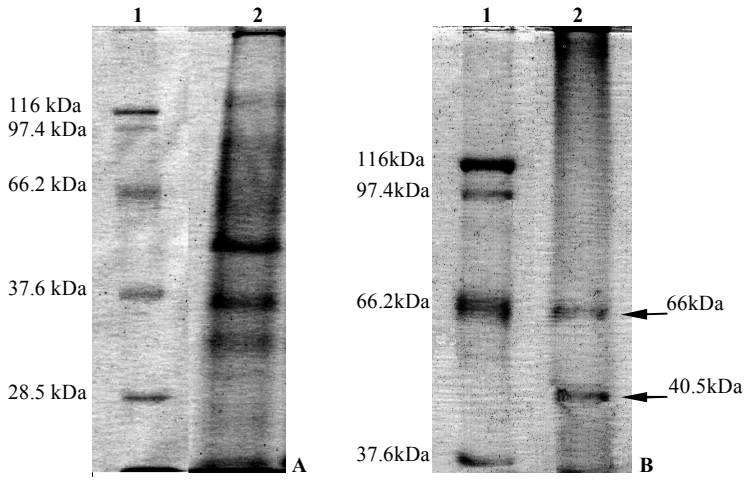

Figure 2. SDS-PAGE of purified α-amylase from *A. niger* 33-19 CNMN FD 02A grown in presence of $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$. **A.** Lane 1: Standard proteins, Lane 2: Ethanol precipitation; **B.** Lane 1: Standard proteins, Lane 2: Ion exchange chromatography sample

DISCUSSIONS

It is well known that addition of metal ions to nutritive medium of microorganisms induce various modifications in proteins structure and their functions. Metal compounds that cause enhancement of biomass or bioactive substances production present significance for biotechnology [18]. Coordination compounds of the metals are widely synthesized and detail explored as the artificial models of metalloproteins/enzymes possessing the potential for future practical applications. However, it is still less known about the mechanism of influence of metal complexes on enzyme synthesis and polypeptide spectrum of microorganisms.

The related method of enhanced productivity of *Spirulina platensis* CNM-CB-02 obtaining with high content of chromium in biomass accumulation by cultivation on nutrient medium supplemented with some coordination compound of Cr(III) confirms the maximum 51-53% distribution of chromium ions in the fraction of proteins and the 15-22 times increase of chromium in biomass. Following comparative study of polypeptide spectrum of total proteins from biomass by SDS-PAGE electrophoresis demonstrate emphasis of two new bands under influence of chromium complexes, with molecular weights of 60.4kDa identified as phytochelatin synthase and 66.7kDa as protein from chaperone family [4].

The investigations of coordination compounds effect on fungi are focused mainly on description of metal complexes with antifungal activity or finding natural synthesized coordination compounds by fungi with practical significance. The studies of mechanisms of stimulation of coordination compounds on fungi hydrolytic enzyme synthesis are very poor.

For the first time this research shows that $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound, proposed as stimulator of amylolytic activity of *A. niger* 33-19 CNMN FD 02A, has an indirect effect on α-amylase synthesis. Polypeptide spectrum of amylolytic preparation obtained from *A. niger* 33-19 CNMN FD 02A micromycete did not modified significantly under the influence of the studied coordination compound. Molecular masses of α-amylase isomorphs identified as 66kDa and 40.5kDa did not modify either.

Thus, compare to earlier work on α-amylase purification from *A. niger* 33-19 CNMN FD 02A presented in [3] we can conclude that $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ coordination compound stimulates amylolytic activity of isolated enzyme preparation with 23%, when specific activity remains actually unchanged. Total amylolytic activity and specific activity of purified α-amylase by extraction with Tris-HCl buffer, gel filtration and ion exchange chromatography decrease significantly, presenting a yield of 13% and a purification coefficient of just 7.8 times.

Amylolytic activity decrease and unchanged polypeptide profile of α-amylase after ion exchange chromatography allows us to suppose that $[Co(DH)_2(An)_2]_2[TiF_6]\cdot 3H_2O$ bind some reactive groups of α-amylase molecule in deficiently purified amylolytic preparation [24]. Coordination complexes may attach to groups from enzymes active site, determining enzyme and substrate binding, or to some additional radicals which are spatially close to active site and may influence their reactive property. The purification of amylolytic preparation could split these bonds, which are considered less strong.

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