Action of Millimeter-range Electromagnetic Radiation on Polypeptide Spectrum of Amylolytic Preparations from *Aspergillus Niger* 33-19 CNMN FD 02A Strain

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Micromycete *Aspergillus niger* 33-19 CNMN FD 02A standard cultivated and exposed to λ = 7.1 millimeter waves radiation has been used to obtain two enzyme preparations with the 10x degree of purity, with a 29.9% difference of the α-amylase activity in irradiated preparation but with a similar specific activity of protein of 138.3 U/mg and 141.0 U/mg. The α-amylase purification through gel filtration and ion exchange chromatography has resulted in the increase of 8.9 times of the specific activity for the control and of 8.2 times for the irradiated samples, with the yields of 25% and 17%, respectively. The SDS-PAGE of proteins isolated from initial preparations indicates the expression of new polypeptide of 45 kDa in the irradiated samples. Polypeptide spectrum of α-amylase from both preparations, purified through ion chromatography, shows two unchanged polypeptide bands of 66 and 40.5 kDa, identified as α-amylase isomorphs.

Keywords: millimeter-range electromagnetic radiation, fungi, Aspergillus niger, α-amylase purification.

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INTRODUCTION

In the past years the electromagnetic radiation (EMR) of the millimeter range has been actively used in biology, medicine, biotechnology, etc. Millimeter waves (MMW) have the wavelength of 10 to 1 mm which runs in the extremely high frequency (EHF) band. The idea that MMW can specifically influence biological structures and whole organisms was first presented by Devyatkov N.D., Golant M.B. and Gel'vich E.A. in 1964–1965 who claimed that this wave frequency can be used by living organisms to transfer information (impulse transmission) between cells [1, 2].

Currently a wide experimental material on the EMR effect of nonthermal intensity on various species of microorganisms, like model-objects of a study: yeasts, bacteria, actinobacteria, cyanobacteria, micromycetes has been gathered, which is helpful in explanation of certain action properties of MMW [3–8]. The evasive influence of the millimeter-range EMR on living cells should be noted, which depends on both frequency and strength of waves and the initial state of a biological object. It is also worth mentioning here that the influence is of a resonant character, i.e. observed in narrow frequency ranges. The main difficulty in the study of the EMR biological effect is that electromagnetic waves overlapping the cell vibrations can induce a positive as well as negative response [9].

The actual mechanism by how EMR generates biological effects is under intense study. Some hypotheses on the MMW action on organisms, which accounts for the fundamentals of their metabolisms, are proposed. It is considered that at the interaction between a millimeter-range electromagnetic radiation and the biological object, main processes take place in cell membranes, converting the electromagnetic energy into acoustic-electric waves and initiating metabolite processes inside the cell. Electromagnetic forces at the surface of a membrane could modify ligand-receptor interactions and, as result, would affect the state of the membrane molecules that control the cell secretion. The electropotential of healthy cells causes a steady flow of ions across the membrane, which is disturbed in a damaged cell. An optimal EMR can restore normal membrane potential, increasing cell activity [10–12].

Herewith, an object for the EMR is cell premembrane water molecules. Such water molecules are essential for biomembrane proteins hydration, switching them from a functionally passive to an active condition. Next, mechanisms that initiated biochemical reactions are activated, for which protein molecules are responsible. Thereby the permeability of membranes increases and leads to the enhancement of the substances transport from the exterior to interior of a cell, changing its biological activity [13, 14].

Our previous studies of the MMW influence on the synthesis of extracellular hydrolases and the life cycle of fungi strains *Aspergillus niger* 33 and *Aspergillus niger* 33–19 CNMN FD 02A – producers of amylases, and *Penicillium viride* CNMN FD 04P – producer of pectinases, show the increase of 44–49% of the enzyme activity at optimal irradiation and the reduction of the growth cycle of *Aspergillus niger* 33–19 CNMN FD 02A within 48 hours. It is considered that irradiation induces structural alterations of cell membranes, causing increase of their permeability and, in turn, an easier enzymes secretion outside the cell [15].

To better understand a possible mechanism of interaction between external MMW and micromycetes – producers of hydrolases, the aim of the given work has been to assess changes in the amylolytic activity and the polypeptide spectrum of enzyme preparation obtained from the micromycete *A. niger* exposed to the millimeter-range electromagnetic irradiation.

EXPERIMENTAL

Microorganism and culture conditions

The object of the study, a strain of the mycelial fungi *Aspergillus niger* 33-19 CNMN FD 02A – producer of amylases, stored at the National Collection of Nonpathogenic Microorganisms at the Institute of Microbiology and Biotechnology of the Academy of Sciences of Moldova, was cultivated in Erlenmeyer flasks of 1000 ml volume, at the temperature of 28–30°C, on shakers (180–200 rpm), during 5 days. Each flask contained 200 ml of a 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. The initial pH of the nutrient medium was 3.0 [16].

Spore suspension of 10% v/v with the density of $3 \cdot 10^6$ spores/ml, obtained by washing with sterile water of the 12–14-days culture, grown on malt-agar oblique columns and irradiated for 15 minutes with electromagnetic radiation in the millimeter range, with the wavelength $\lambda = 7.1$ mm in a constant mode, served as the seed material [17]. As a source of a low-intensity EMR, a UEMA-3 (Moldova) instrument was used. Samples with nonirradiated inoculum were taken as the control.

Enzyme extraction

The media of the 144-hours grown control culture and the 96-hours grown irradiated culture were separated from biomass by filtration and 20-minutes centrifugation at 4000–6000 rpm. The α-amylase was isolated from the supernatant with 96% ethanol cooled to -15°C, in the ratio 1:4 C_2H_5OH, with the sedimentation duration of one hour and the concentration of $CaCl_2$ 0.2%. The sediment was centrifuged for 20 minutes at 6000 rpm and dried at 20–22°C [18]. Thus obtained enzyme preparation was further investigated and purified.

Enzyme assay

The α-amylase activity was determined according to the SKB colorimetric method by measuring the amount of the different released molecular weight dextrins at 30% hydrolysis of one gram of a soluble starch after 10 minutes incubation at 30°C. The reaction mixture (15 ml) contained 10 ml of buffered (0.2M acetate buffer, pH 4.7) 1% soluble starch (Sigma) solution as the substrate and 5 ml adequately diluted enzyme sample. The reaction was stopped and developed with iodine solution in the ratio of 0.5 ml reaction mixture/50 ml iodine solution (5 mg iodine and 50 mg KI in 100 ml 0.1N HCl) for 5 minutes [19].

Protein assay

Total protein content was determined according to the Lowry method of taking crystalline bovine serum albumin as the standard [20].

α-Amylase purification

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

Ion exchange chromatography. Polled fraction was loaded onto an anion exchange HiTrap™ Q (5 ml) column at the flow rate of 1 ml/min in the FPLC System (Amersham Pharmacia). The column was equilibrated with the 20 mM Tris-HCl buffer. The bound 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.

Electrophoresis

To evaluate purity of α-amylase isolated proteins from amylolytic preparations and from amylolytic active fractions were applied to 15% SDS-PAGE electrophoresis, according to the Laemmli method [21]. The gel was stained with coomasie Brillant Blue R-250. Molecular weight markers were β-Galactosidase (116 kDa), Phosphorylase b (97.4 kDa), Bovine serum albumin (66.2 kDa), Alcohol dehydrogenase (37.6 kDa), Carbonic anhydrase (28.5 kDa).

Statistical analysis

All experiments were performed three times and the results are presented as the simple mean of three, with the level of significance $p \leq 0.05$ [22].

RESULTS

Enzyme complex isolation from cultural liquid of the *A. niger* standard grown (I) and irradiated with the millimeter-range electromagnetic radiation (II) allowed for obtaining two amylolytic preparations of the 10x degree of purity (Table 1). The preparation from the irradiated *A. niger* had a 29.9% higher α-amylase activity and 27.6% higher protein amount. Due to the increase of the total protein, the specific activity of both preparations was similar, i.e. 138.3 U/mg and 141.0 U/mg proteins, respectively.

Table 1. Amylolytic properties of enzyme preparations obtained from *A. niger*

Variants	Protein (%)	α-Amylase activity (U/g)	Specific activity (U/mg)
A. niger 33-19 grown under standard conditions (I)	23.5	32554.5	138.3
Irradiated with λ = 7.1 mm culture of *A. niger* (II)	30.0	42314.0	141.0

The extraction of α-amylase from both enzyme preparations and their purification by gel filtration and ion exchange chromatography gave an increase of the enzyme specific activity of 8.9 times for the control and 8.2 times for the preparation obtained at *A. niger* spore suspension irradiation with MMW (Table 2). The yield of α-amylase was 25% for the control and 17% for the irradiated samples (1.5 times less), showing a greater decrease of total amylolytic activity of the irradiated preparation after purification. The protein amount of both purified preparations was identical, of 0.160–0.162 mg/ml.

In both cases, ion chromatography of protein separated from the preparations under study (Fig. 1) shows four fractions with amylolytic activity and a peak in one fraction, number 18 for the standard cultivated sample and number 14 for the irradiated sample. The general elution profile of the proteins from enzyme preparations is different. For the control sample, the peak of the α-amylase activity coincides with the peak of the protein amount. For the irradiated sample, the peak of the α-amylase activity does not coincide with the peak of protein, although the values of both amylolytic activity and protein content are practically similar in both cases. A hig-her protein content was observed in fraction 1 (0% NaCl gradient) and fraction 10 (32% NaCl), as 53.7% and 29.2%, respectively. Presented data are recalculated for 1.5 times dilution, used for the solution applied on ion chromatography.

SDS-PAGE electrophoresis of proteins isolated from *A. niger* enzyme preparations indicates a high heterogeneous protein content of initial complexes and some difference in polypeptide bands (Fig. 2A). A major difference is that MMW induce expression of a new polypeptide with an apparent molecular weight of 45 kDa, absent in the control sample.

The polypeptide spectrum of the determined amylolytic active fractions after ion exchange chromatography shows two polypeptide bands with apparent molecular weights of 66 kDa and 40.5 kDa after purification (Fig. 2B). The appeared polypeptides are considered as 2 isomorphs of α-amylase [23] and demonstrate that the applied λ = 7.1 millimeter-range electromagnetic radiation does not induce structural changes in α-amylase of the *A. niger* strain.

DISCUSSIONS

It is generally accepted that living organisms are not indifferent to electromagnetic radiation of millimeter range and a proper application of the EHF-radiation can be used to stimulate and regulate various cell processes. Yet the biostimulating phenomena have no comprehensive explanation just some hypotheses are discussed. It is certain that besides similarities of the millimeter-range radiation effects on various photosynthetic and non-photosynthetic organisms, some differences at fungi investigation can be identified: frequent morphological changes of cells, active wavelengths 4.9, 5.6, 5.95–7.2 mm, the intensification of a process with the simultaneous slowing down of another one, multiple MMW exposures of fungi spores (repetition factor of 10 times) so as to get a stable effect, inheritance of acquired changes by further generations [11, 15].

Although the crucial role of proteins in every biological process is known and some hypotheses of the EHF-radiation influence on proteins were proposed (increase of protein molecules hydration, magneto-reception of some ions bound with proteins etc.), studies in this field are still insufficient or completely missing when talking about mycelial fungi – producers of biotechnologically important enzymes.

The given research shows that the electromagnetic radiation of a millimeter range, chosen as a stimulator of enzyme synthesis by micromycete of *A. niger*, does not induce direct changes in the structure of α-amylase. Standard cultivated samples and the ones exposed to the optimal MMW of λ = 7.1 gave similar polypeptide bands after ion exchange purification, corresponding to the two isomorphs of α-amylase, of 66 kDa and 40.5 kDa.

MMW do not affect α-amylase, but induce other changes in the polypeptide spectrum of proteins released outside the cells in the culture medium of *A. niger*. A biological effect of the millimeter-range EMR on the irradiated preparation is demonstrated

Table 2. Purification profile of α-amylase from amylolytic preparations obtained under standard conditions (I) and at irradiation with MMW λ = 7.1 of *A. niger* (II)

Purification Steps	α-Amylase activity (U/ml)		Protein (mg/ml)		Specific activity (U/mg)		Yield (%)		Purification fold	
	I	II	I	II	I	II	I	II	I	II
Amylolytic preparation solution (25 mg/ml)	813.86	1057.85	5.875	7.500	138.30	141.00	100		1	
Extract	422.88	709.12	1.908	2.487	221.63	285.13	52	67	1.6	2.0
PD-10 column gel filtration	390.12	590.38	1.173	1.725	332.45	342.25	48	56	2.4	2.4
HiTrap™ Q column ion exchange chromatography	199.68	184.98	0.162	0.160	1232.61	1156.13	25	17	8.9	8.2

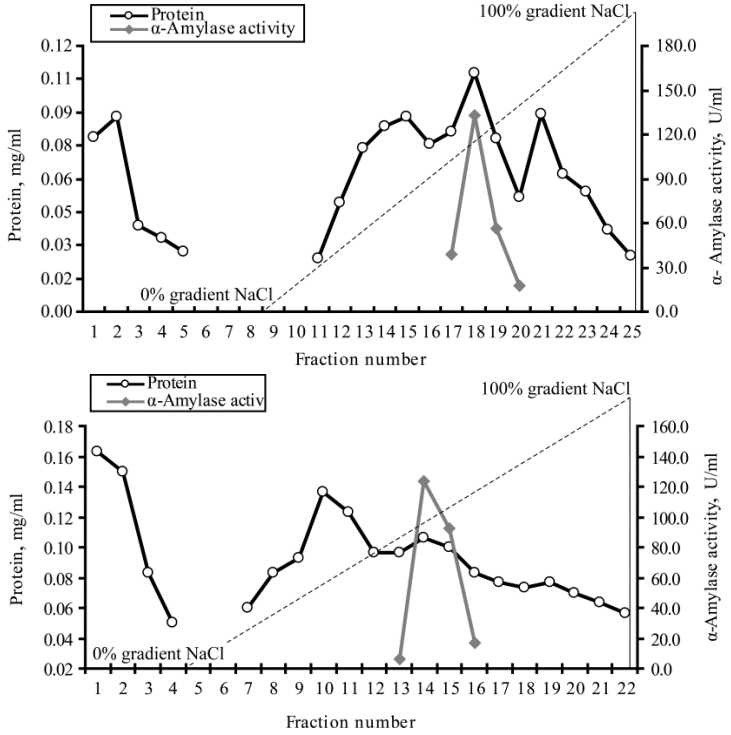

Fig. 1. Purification of α-amylase from amylolytic preparations obtained under standard conditions (I) and at irradiation with MMW λ = 7.1 of *A. niger* (II) by ion exchange chromatography. Samples: 2 ml of α-amylase preparation after gel filtration through PD-10 column. Column: HiTrap™ Q (5 ml). Flow rate: 1 ml/min. Buffer A: 20 mM Tris-HCl, pH 7.0. Buffer B: A+0.5M NaCl. Gradient: for I 0% B in 27 ml, 0%–100% B in 75 ml and for II 0% B in 15 ml, 0%–100% B in 66 ml.

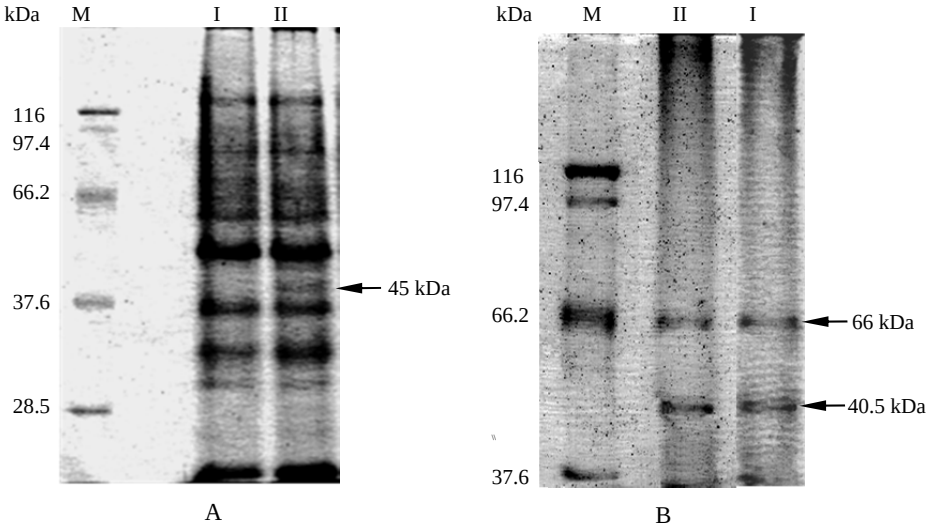

Fig. 2. SDS-PAGE of *A. niger* α-amylase purified through ethanol precipitation (A) and ion exchange chromatography (B). Lane M: Protein markers, Lane I: Sample obtained from *A. niger* cultivated under standard conditions, Lane II: Sample obtained at *A. niger* irradiation with millimeter wavelength range λ = 7.1.

by expression of a new polypeptide band with the apparent molecular weight of 45 kDa, and, also, by differences in the elution profile of proteins from both the control and irradiated preparations, where peak of the protein content does not coincide with the peak of the α-amylase activity, as in the control sample.

How the millimeter-range EMR causes changes in the polypeptide spectrum of proteins isolated from the culture liquid of the irradiated *A. niger* still need to be discussed. According to one of the hypotheses, it is supposed that MMW increases permeability of cells membranes, leading to accumulation of a "non-specific" protein outside the cell. This modification could change amylolytic activity of the isolated enzyme preparation that was by 29.9% higher in the non-purified irradiated preparation and, practically, similar after ion exchange chromatography, compared to the control preparation.

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Реферат

Из мицелиального гриба *Aspergillus niger* 33-19 CNMN FD 02A, культивируемого в стандартных условиях и после облучения миллиметровыми волнами λ = 7,1, были получены два ферментных препарата со степенью очистки Г10х, отличающихся по уровню амилолитической активности (на 29,9% выше в препарате, подвергнутом облучению), но с идентичной специфической активностью в 138,3 ед/мг и 141,0 ед/мг белка. Очистка α-амилазы методами гель-фильтрации и ионообменной хроматографии привела к увеличению специфической активности в 8,9 раз в контрольном и в 8,2 раза в облученном препаратах, с 25% и 17% выходом, соответственно. SDS-ПААГ белков, выделенных из начальных препаратов, показала появление нового полипептида с молекулярной массой 45 kDa в образце, подвергнутом облучению. Полипептидный спектр очищенной α-амилазы обоих препаратов после ионной хроматографии представляет 2 неизменные полипептидные полосы с молекулярной массой 66 и 40,5 kDa, определенных как изоформы α-амилазы.

Ключевые слова: электромагнитное излучение миллиметрового диапазона, мицелиальные грибы, Aspergillus niger, очистка α-амилазы.