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Isolation, Purification, and Biological Activity of Secondary Metabolites from *Trichoderma asperellum* F-1087

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Abstract—The secondary peptide metabolites produced by the *Trichoderma asperellum* strain F-1087 were isolated, and their properties were studied. It has been shown these metabolites at concentrations of 0.08 and 0.02 mg/mL inhibit the human prostate cancer cell line by 97 and 34%, respectively.

Keywords: secondary metabolites, *Trichoderma asperellum*, PC-3
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INTRODUCTION

The genus of fungi *Trichoderma* is widely distributed in different climatic zones; they occupy a variety of habitats. These fungi can be isolated from a large number of natural and artificial substrates [1, 2]. The survival of these fungi in different climatic zones is caused by their high adaptability, as well as the wide metabolic variety of strains, their high reproductivity, and their competitiveness [3].

Mycelial fungi, including molds, are effective producers of secondary metabolites (products of the producer's vital activity) that possess biological activity and influence the environment of the producer by suppressing the growth of other microorganisms [4–7]. Many secondary metabolites do not have a definite function and are often synthesized under certain cultivation conditions, for example, stressful ones [8, 9]. Some of them are of interest to the pharmaceutical industry (enzymes and antibiotics) [10, 11].

The purpose of this work was to isolate and characterize the secondary peptide metabolites of the mold fungus *T. asperellum* F-1087.

MATERIALS AND METHODS

The object of the study was the *Trichoderma asperellum* F-1087 strain isolated from the soil of the Murzikhinsky II burial ground (Republic of Tatarstan, Russia). The strain was deposited in the All-Russia Collection of Industrial Microorganisms under the registration number F-1087.

To obtain the seeding material, the fungus *T. asperellum* F-1087 was grown on Czapek nutrient medium of the following composition (g/L): glucose 30, $NaNO_3$ 3.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.01, and agar-agar 15. Cultivation was carried out in the dark for 14 days at 28°C [12]. After 14 days of growth, the formation of a dark green pigment was observed.

Mycelium with a portion of the solid medium was placed in a liquid nutrient medium of the following composition (g/L): glucose 5, KH_2PO_4 0.8, KNO_3 0.7, $CaCO_3$ 0.06, $MgSO_4$ 0.5, $ZnSO_4$ 0.01, $CuSO_4$ 0.005, $FeSO_4$ 0.001, $MnSO_4$ 0.01 and distilled water 1 L; H_3PO_4 was added to a concentration of 1.75%; and mycelium was cultured in 1000-mL conical flasks containing 500 mL of the medium for 12 days on a Helicon shaker (Germany) at a platform rotation speed of 80 rpm at 28°C. During cultivation of the fungus, the product yield, the morphological state of the culture, and the absence of extraneous microflora were monitored.

After the cultivation was completed, the culture liquid (CL) of *T. asperellum* F-1087 was separated by filtration through a layer of gauze and centrifuged at 4800 *g* for 15 min. Ethyl acetate (Khimmed, Russia) was poured onto the resulting supernatant in a 1 : 1 ratio, stirred, and incubated for 24 h at 4°C. In this case, a clear separation of the boundaries of the two phases was observed. The upper phase (ethyl acetate) was evaporated to a volume of 1 mL on a Heidolph vacuum rotary evaporator (Germany) for 4 h at 30°C and then dissolved in 50 mL of acetonitrile (Merck, Germany).

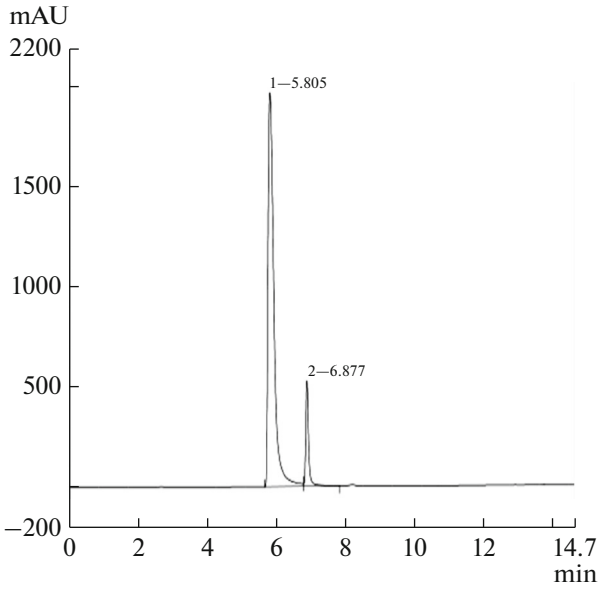

Fig. 1. Reversed-phase HPLC of ethyl acetate extracts from cultural liquid of *T. asperellum* F-1087.

Biuret and xantoprotein reactions (Mulder's reaction) were carried out to qualitatively identify metabolites.

Metabolites were further purified by reversed-phase high performance liquid chromatography (RP-HPLC) with a Dionex chromatograph (United States) on a C-18 Agilent column (United States), which was conducted according to protocol [13]. The column was equilibrated with acetonitrile for 10 min at a flow rate of 0.4 mL/min, and the ethyl acetate phase (2 μL) was then injected. The elution of the metabolites contained therein was carried out with a concentration gradient from 100% acetonitrile to 100% Milli-Q H_2O for 10 min at a flow rate of 0.4 L/min. Detection was carried out at 220 nm, which corresponds to the maximum absorption of peptides. Two components were collected and used for further characterization. The collection of fractions was repeated 50 times to accumulate a sufficient amount of metabolites.

The extracted metabolites were analyzed on a Bruker mass spectrometer (United States) by the MALDI-TOF/TOF method in MS mode. Positively charged ions were evaluated in the reflex mode in the range of 600–1500 *m/z*. For analysis, fractions after HPLC were preconcentrated tenfold; 2 μL of the fraction was mixed with 0.5 μL of a saturated matrix solution. A solution containing 30% 2,5-dihydroxybenzoic acid, 69% acetonitrile, 5% water, and 0.5% trifluoroacetic acid served as a matrix.

The metabolic activity of peptide metabolites was studied in the PC-3 cell lines (human prostate cancer cells) [14]. The PC-3 cell lines were cultured in α-MEM medium containing 10% phosphate buffer, 1 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a CO_2-incubator in 5% CO_2 atmosphere, with replacement of the medium every 3–4 days. The PC-3 cells (3×10^4 cells/mL) were added to the wells of a 96-well plate. After 24 hours of incubation, the nutrient medium was replaced with a medium containing different amounts of isolated and purified peptide metabolites. To do this, various concentrations of the metabolite were dissolved in aqueous acetonitrile: 1.7, 0.08, 0.04, 0.02, 0.01, 0.005, 0.002, 0.001, 0.0006, and 0.0003 mg/mL. The amount of aqueous acetonitrile in each well did not exceed 10% of the total volume. Cells in the presence of metabolites were incubated for 72 h at 37°C. The culture medium was then replaced by a single phosphate buffered saline (pH 7.4) containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium bromide (MTT). After incubation in the medium containing MTT, dimethyl sulfoxide was replaced to dissolve formazan, a product of MTT reduction by viable cells, which was recorded on a microplate reader (Tecan, Switzerland) at 555 nm (maximum absorption of formazan) and adjusted for plastic. Due to differences in the thickness of the bottom of the wells, we used the maximum, taking into account the absorption of plastic at 750 nm. The number of viable cells was calculated relative to control (untreated cells). The amount of peptide metabolites that inhibited cell growth by 50% relative to control was taken as IC_{50}. In the study of cytotoxicity against the PC-3 cell line, the initial amount of the secondary metabolite was 1.7 mg/mL. Survival of the PC-3 cell lines was calculated by the formula optical adsorption of peptide / mean value of optical adsorption of the control.

RESULTS AND DISCUSSION

Metabolites were extracted from CL of *T. asperellum* F-1087 with ethyl acetate; the components contained in the extract were separated by RP-HPLC. In this case, two components were detected (Fig. 1). Their retention times made it possible to conclude that these components had a hydrophobic elution profile characteristic of peptide metabolites [23]. A positive biuret reaction with these components confirmed that the studied metabolites had a peptide nature. The mass spectrometric analysis of the extract from the fungal CL (Fig. 2) showed the presence of peaks in the range of mass-to-charge (*m/z*) values from 700 to 1500. With further fragmentation of the metabolite (*m/z* 885.533), 55 possible variants of the amino acid composition of the peptides were found. These variants, in decreasing order of the likelihood of their presence in CL of *T. asperellum* F-1087, were as follows: VRRRKNG, GNZMHWL, TSEHLWL, TGPRRWL, TSELHWL, GPTRRWL, RVRRKNG, GNZMHLW, GNCHRWL, GNCRHWL, GNMZHWL, GNHCRWL, GNRCHWL, MWLHKNG, WMCFKNG, MWCFKNG, WMLHKNG, WMSYKNG, MWSYKNG,

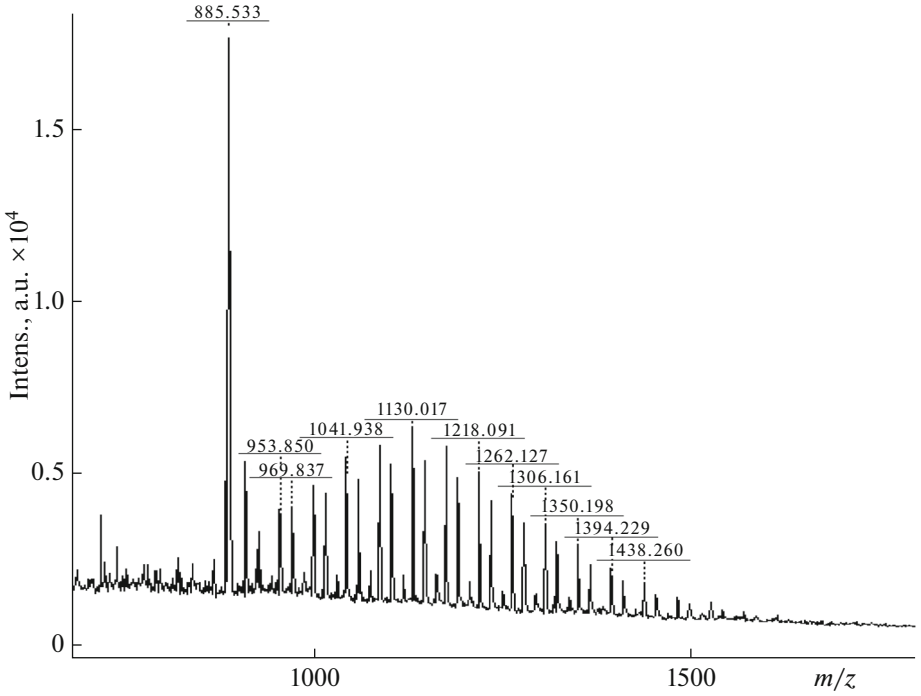

Fig. 2. Mass spectrum of metabolites isolated from cultural liquid of *T. asperellum* F-1087.

TSELHLW, GNRCHLW, GNCRHLW, GNMZHLW, PGTRRWL, GTPRRWL, GPYLHWL, GPYHLWL, GVVRRWL, VGVRRWL, PGYLHWL, RVRRWL, AALRRWL, PGYHLWL, GPYLHLW, YPGLHWL, YPGHLWL, GLFLHWL, GLFHLWL, GEMLHWL, GEMHLWL, GMELHWL, GMEHLWL, GFLLHWL, GFLHLWL, GYPLHWL, GYPHLWL, YGPLHWL, YGPHLWL, GFLLHLW, GYPLHLW, YGPLHLW, GEMLHLW, YPGLHLW, GMELHLW, and GLFLHLW.

Ethylnorvaline (Z), a nonproteinogenic amino acid, could presumably be a part of some peptides; this amino acid is a characteristic component of peptaibols. Peptaibols are short peptides containing 7–20 amino acid residues, among which there are nonproteinogenic α-aminoisobutyric acid, ethylnorvaline, isovaline, or hydroxyproline [23]. A search of the Peptaibol Database (http://peptaibol.cryst.bbk.ac.uk) showed that the isolated peptides do not belong to the known peptaibols. It can be assumed that they are related to the not yet identified peptaibols. Secondary metabolites of a similar species of *T. asperellum* were studied previously; their mass spectrometric identification showed that, in the CL composition, there were peptaibols of the group of asperylin and trichotoxin possessing antibacterial, antifungal, and antiviral activity [20, 23].

The effect of peptaibols of *T. asperellum* F-1087 on eukaryotic cells of the PC-3 line was studied. It turned out that they inhibited the metabolic activity of the cells. Figure 3 shows that the death of PC-3 cell lines is directly proportional to an increased peptaibol content in the wells. Maximum inhibition (90%) was observed at a metabolite concentration of 1.7 mg/mL. The concentration at which 50% of the cells died (IC_{50}) was 0.03169 mg/mL. These data supplement the results of our previous work, in which the effect of secondary metabolites of *T. asperellum* F-1087 on cervical and breast cancer cell lines was studied [14].

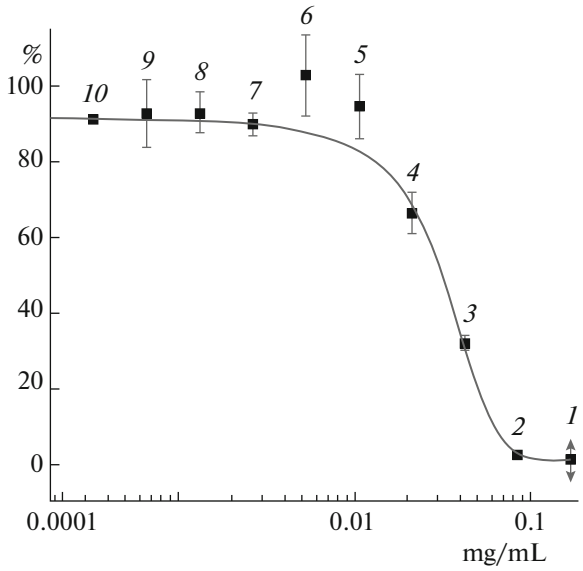

Fig. 3. Viability (%) of PC-3 cells under the action of secondary metabolites from *T. asperellum* F-1087 at concentrations of (*1*) 1.7, (*2*) 0.08, (*3*) 0.04, (*4*) 0.02, (*5*) 0.01, (*6*) 0.005, (*7*) 0.002, (*8*) 0.001, (*9*) 0.0006, and (*10*) 0.0003 mg/mL (MTT assay).

As a result, it has been found that the secondary metabolites isolated from CL of *T. asperellum* F-1087 have a peptide nature similar to peptaibols. Secondary metabolites of this mold fungus can suppress a wide range of human cancer cell lines.

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