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A NEW TOXIC PROTEIN FROM DEATH CAP *AMANITA PHALLOIDES*: ISOLATION AND STUDY OF CYTOTOXIC ACTIVITY

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Cytotoxic protein from fruit body of the death cap *Amanita phalloides* mushroom, designated as toxophallin, has been isolated and its principal physico-chemical and biological properties have been characterized. This protein possesses molecular mass 55 kDa and isoelectric point at pH 5.7 and is expressed predominantly in the stem of mushroom fruit body. As assessed by cross-linking experiments, toxophallin forms a trimeric complex in buffered solution. It induces apoptosis in several mammalian cells lines (murine L1210 and CCL-64, and human A549) with IC₅₀ at 0.25–0.45 µg/ml. It also induces DNA fragmentation and morphological changes in the nuclei of target cells (chromatin condensation and fragmentation of nuclei) that are characteristic for apoptosis. Caspase III Inhibitor (Boc-D-fmk) does not inhibit toxophallin-induced apoptotic DNA fragmentation, suggesting that this toxin involves caspase-independent pathway of apoptosis. Besides, it was shown that toxophallin interaction with target cells is not mediated by specific cell surface receptor. Thus, toxophallin is a new toxic protein whose properties distinguish it from other toxic compounds (cyclopeptides and phallolysin) earlier found in the death cap.

Key words: toxic protein, death cap *Amanita phalloides*, apoptosis.

The abbreviations used in the article:

Boc-D-fmk, Benzyloxycarbonyl-Asp-Ome-fluoromethylketone, Caspase Inhibitor III;

DAPI, 4,6-diamidino-2-phenylindole;

DMEM, Dulbecco's modified Eagle's medium;

PBS, phosphate-buffered saline;

TUNEL, terminal deoxynucleotidyl-transferase (TdT) mediated dUTP-biotin nick end labeling.

НОВИЙ ТОКСИЧНИЙ БІЛОК ІЗ БЛІДОЇ ПОГАНКИ *AMANITA PHALLOIDES* L.: ОЧИСТКА ТА ДОСЛІДЖЕННЯ ЦИТОТОКСИЧНОЇ АКТИВНОСТІ

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*Кому скеровувати кореспонденцію

Із фруктового тіла гриба блідої поганки *Amanita phalloides* L. очищено токсичний білок, названий токсософаліном. Описано його основні фізико-хімічні та біологічні властивості. Цей білок має молекулярну масу 55 кДа й ізоелектричну точку при рН 5.7. Він зосереджений переважно в основі фруктового тіла гриба. Експерименти із поперечною зшивкою білкових молекул продемонстрували, що токсософалін утворює тримерний комплекс у буферному розчині. Він індукуює апоптоз у ряді ліній клітин ссавців (мишачі клітини ліній L1210 і CCL-64, а також людські клітини лінії A549) із IC₅₀ при 0.25–0.45 мкг/мл. Він також індукуює фрагментацію ДНК та морфологічні зміни в ядрі клітин-мішеней (конденсацію хроматину і фрагментацію ядра), що характерно для апоптозу. Інгібітор каспази III (Вос-D-fmk) не пригнічував апоптичної фрагментації ДНК, що дає змогу припустити, що цей токсин залучає в дію каспазо-незалежний шлях апоптозу. Крім того, виявлено, що взаємодія токсософаліну з клітинами-мішенями не опосередковується специфічними рецепторами на поверхні цих клітин. Отже, встановлено, що токсософалін є новим токсичним білком, властивості якого відрізняють його від інших токсичних сполук (циклопептиди і фалолізін), знайдених раніше у блідій поганці.

Ключові слова: токсичний білок, бліда поганка *Amanita phalloides*, апоптоз.

INTRODUCTION

Death cap (*Amanita phalloides* L.) is known as a deadly poisonous mushroom due to the presence of several toxic substances. First of them was isolated in 1937 by Wieland H. (see: [1]) and identified as oligopeptide. In further works, Wieland Th. revealed other toxic cyclopeptides which were classified into two structural groups – amanitine and phalloidine, exhibiting different mechanisms of toxic action [21]. While amanitin is blocking mRNA transcription, phalloidin binds to actin and alters cytoskeleton functions. These cyclopeptides are frequently used as tools in scientific studies since their intracellular molecular targets and mechanisms of action are well characterized. It should be noted that besides toxic peptides this mushroom also contains antitoxin antamanide, a cyclodecapeptide which blocks phalloidin effects [1, 21]. Later on, another toxic polypeptide substance, phalloysin, possessing hemolytic activity was detected in the fruit bodies of the death cap [4, 5, 16–18]. Its chemical properties and biological activity, as well as the mechanism of action, are well studied [8, 21].

Many mushroom species were shown to contain substances that possess immunomodulating and antitumor activity [3, 9, 13]. Lectin-like proteins demonstrating antiproliferative activity towards tumor cells were isolated from *Tricholoma mongolicum* [20] and *Agaricus bisporus* [15] mushrooms. Another antineoplastic protein, volvarin, that belongs to the family of ribosome inactivating proteins (RIP) type I, was isolated from edible mushroom *Volvariella volvacea* [22]. Poisonous mushroom *Boletus satanas* Lenz contains a toxic lectin bolesatine which inhibits protein synthesis *in vitro* and *in vivo* [10].

In the present work, we have characterized a novel protein isolated from fruit bodies of the death cap. An improved procedure for purification of this protein has been described. Detailed investigation of its action towards several lines of tumor cells was carried out. Its physico-chemical and biological properties clearly distinguish this novel cytotoxic protein from phallolysin [12].

MATERIALS AND METHODS

Isolation and Purification of Cytotoxic Proteins from the Death Cap

Fruit bodies of *Amanita phalloides* mushrooms were collected in the forests of the Lviv Region (Ukraine), and stored at -20°C until use (no longer than 2 weeks). Thawed mushrooms were pressed, subjected to centrifugation for 15 min at 4,000 g, and the supernatant was collected. Ammonium sulfate was added to the supernatant to 90% saturation, and precipitated proteins were collected by filtration. For elimination of dark colored pigment, the precipitate was dissolved in a small volume of distilled water, dialyzed against buffer solution (50 mM potassium phosphate buffer, pH 7.0 supplemented with 100 mM sodium chloride), and passed through a DEAE-cellulose column (Serva), equilibrated with the same buffer. The fraction of unabsorbed protein was collected and precipitated with ammonium sulfate at 90% saturation.

For elimination of cytolytic lectin, phallolysin, the crude protein fraction was passed through a column filled with affinity sorbent – ovomucin immobilized on agarose [2], that was equilibrated with PBS. Unbound „non-lectin” protein fraction was collected, dialyzed against 30 mM sodium acetate buffer (pH 5.3), and applied onto a CM-cellulose column (Whatman, CM-32), equilibrated with 30 mM sodium acetate buffer, pH 5.3. The absorbed protein material was eluted in two steps: first with 100 mM sodium acetate buffer, and subsequently with the same buffer supplemented with 75 mM sodium chloride. Protein possessing cytotoxic activity was eluted with 100 mM sodium acetate buffer, pH 5.3, containing 75 mM sodium chloride. This protein peak was collected, concentrated, and subjected to re-chromatography on the CM-cellulose column in 100 mM sodium acetate buffer, pH 5.3, with 75 mM sodium chloride. The main protein peak corresponding to pure cytotoxic protein, was collected, dialyzed against distilled water, and lyophilized.

Electrophoretic Study

Two electrophoretic systems were used for protein characteristics: 1) disc-electrophoresis in 7.5% polyacrylamide gel (PAGE) using the Reisfeld system in β -alanine-acetate buffer, pH 4.5 and protein staining with Amido Black 10 B [14], and 2) SDS-PAGE in 14% slab gel in Laemmli buffer system [11] and followed by protein visualization using Coomassie Brilliant Blue R 250. Protein markers of molecular mass were ranged from 14.4 to 94 kDa (GE Healthcare).

Isoelectric Point (pI) Determination

Analytical isoelectric focusing was performed on Immobiline DryStrip pH 3–10, 18 cm, linear (Amersham Pharmacia Biotech, Uppsala, Sweden) in IPGphor (GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations. After resolution in the second dimension by SDS-PAGE in 10% gel, proteins were visualized by silver staining and pI was determined according to the manufacturer's instructions.

Cells

Human lung carcinoma epithelial A549 cells, mink lung epithelial CCL-64 cells, human breast adenocarcinoma MCF-7 and T47D cells, and murine leukemia L1210 cells were obtained from American Type Culture Collection (Manassas, USA). Cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 50 µg/ml of streptomycin.

Toxophallin Iodination and Cross-linking

Toxophallin was iodinated using Chloramine-T method, as described by Frolik et al. [7], and cross-linking with BS³ (Bis(sulfosuccinimidyl)suberate, Pierce) was performed, as described in [6]. The binding experiments were performed at 4°C to avoid potential internalization of the bound protein.

[³H]-Thymidine Incorporation Assay

[³H]-thymidine incorporation into DNA of target cells grown at different concentrations of toxophallin, was determined as described in [19].

DNA Fragmentation Assay

Total genomic DNA from treated cells was extracted, as described in [8], and analyzed by electrophoresis in 1% agarose gel after DNA visualization by the ethidium bromide.

Study of Apoptotic Cells by Fluorescence Microscopy

Morphology of target cell nucleus and its changes under toxophallin treatment were examined by fluorescence microscopy using an Axioplan 2 microscope (Zeiss, Jena, Germany), after cell staining with DAPI (4,6-diamino-2-phenylindole), as described in [19].

DNA fragmentation in individual cells was studied by TUNEL assay (terminal deoxynucleotidyl-transferase (TdT) mediated dUDP nick end labeling) using In Situ Cell Death Detection Kit, Fluorescein (Boeringer Mannheim), according to the manufacturer's instructions.

Statistical Analysis

All experiments were repeated at least three times with a minimum of three parallels. Standard deviation was calculated, and statistical significance of difference was evaluated by using Student's t-test ($P < 0.05$).

RESULTS

Purification of Toxophallin

The purification procedure consisted of 4 steps: 1) ammonium sulfate precipitation of total protein from juice of thawed and grinded mushrooms; 2) elimination of pigment(s) from the obtained protein bulk by ion-exchange chromatography on DEAE-cellulose column; 3) affinity chromatography on absorbed immobilized ovomucin for removing cytolytic lectin, phalloysin; 4) purification of toxophallin by repeated ion-exchange chromatography on CM-cellulose column. The first three steps were

essentially the same, as described in [12]. As an additional step, re-chromatography on CM-cellulose column was introduced for achievement of higher grade of toxophallin purification. Described procedure permitted to obtain toxophallin preparation showing more than 95% purity. Protein yield at sequential purification steps is demonstrated in Table 1. Since it was difficult to compare cytotoxic activity of protein fractions at different steps of purification procedure (Table 1), we can only suggest that fold of toxophallin purification was about 70.

Table 1. Protein yield during the course of toxophallin purification

Step	Characteristics of purification stage	Protein yield, mg
1	Protein precipitation of 500 ml of juice of death cap fruit bodies by ammonium sulphate at 90% saturation	650
2	Elimination of pigments by DEAE cellulose chromatography	150
3	Elimination of phallolysin by affinity chromatography on ovomucin-conjugated agarose	110
4	Ion-exchange chromatography on CM-cellulose	15
5	Re-chromatography on CM-cellulose	9

Electrophoresis at non-denaturing conditions (pH 4.5) of water-soluble protein sample after elimination of pigment revealed 3 main protein bands (Fig. 1). The predominant band corresponds to phallolysin which is known to exhibit high cytolytic activity. Affinity chromatography on immobilized ovomucin sorbent was used for effective removing phallolysin which specifically binds to the ovomucin, while other proteins do not possess such affinity.

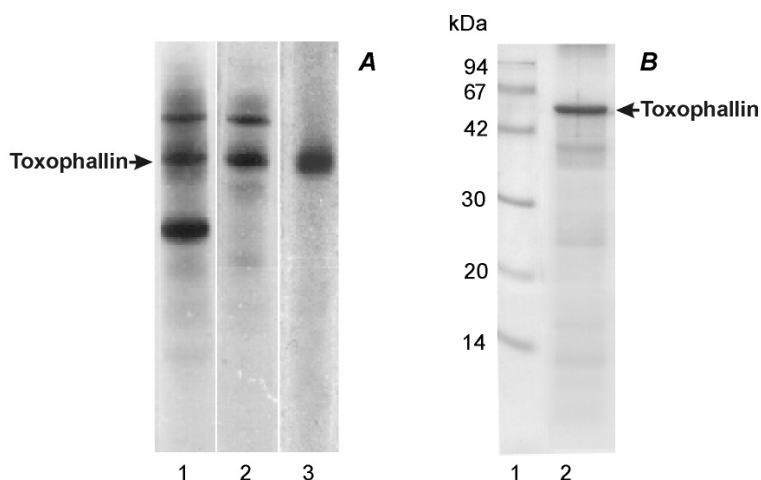


Fig. 1. Electrophoretic study of extracted proteins of *Amanita phalloides*.

A – β -alanin-acetate electrophoretic system, pH 4.5:

1) crude extract; 2) non-lectin proteins (not retained by affinity sorbents); 3) cytotoxic protein purified by ion-exchange chromatography on CM-cellulose column.

B – SDS-PAGE in 14% gel:

1) molecular mass protein markers (Sigma); 2) purified protein (55 kDa) under study. Coomassi R-250 staining

Cytotoxic activity of the obtained fractions was monitored by testing hemolytic and cytotoxic effects towards L1210 leukemia cells *in vitro*. Only traces of hemolytic activity were found in non-absorbed fraction (400 µg/ml), while the hemolytic activity of pure phallolysin was equal 5 µg/ml. Cytotoxic activity was detected not only in phallolysin fraction retained on the column and eluted thereafter, but also in non-retained (non-lectin) fraction.

Protein exhibiting cytotoxic activity found in the non-lectin fraction, was further purified by the ion-exchange chromatography and re-chromatography on CM-cellulose column (see: Materials and Methods). Toxophallin was eluted from the column by 100 mM sodium acetate buffer supplemented with 75 mM sodium chloride. Purified toxophallin migrated as a homogenous band at electrophoresis under the non-denaturing conditions (Fig. 1, A). Single protein band of 55 kDa was also detected at SDS-PAGE in the presence or absence of reducing agent (Fig. 1, B, data not shown). The purity of toxophallin preparation was about 95 % according to the results of electrophoresis in non-denaturing conditions, and about 85 % at SDS-PAGE. We suggest that minor Coomassie-stained bands with the molecular mass below 55 kDa might be products of the proteolytic degradation of the main protein band.

The isoelectric point (pI) of purified toxophallin measured by means of 2D-electrophoresis using Immobililine DryStrip pH 3–10, was found to be pH 5.7.

Amino Acid Composition

Amino acid analysis showed 3 cysteine, 6 methionine and 36 proline residues in toxophallin molecule that is about 7 % of amino acid residues present in this 55 kDa protein comprising of about 503 amino acid residues (Table 2). Relatively high content of proline residues suggests a significant rigidity of polypeptide chain of the toxophallin.

Biological activity of toxophallin

Toxophallin possesses cytotoxic activity that was detected by trypan blue exclusion assay in L1210 murine leukemic cells *in vitro*. IC₅₀ of partially purified toxophallin (non-lectin protein fraction) was equal 0.35 µg/ml, while after final purification step that indicator was equal 0.28 µg/ml. There was no cytolytic activity in the purified toxophallin preparations (data not shown).

To explore whether toxophallin exhibits a specific effect towards tumor cells as compared to cells obtained from normal tissues, [³H]-thymidine incorporation assay was applied. It was revealed that toxophallin-induced inhibition of [³H]-thymidine incorporation by target cells was similar in different cell lines tested (Fig. 2). IC₅₀ ranged from 0.25 µg/ml in human lung carcinoma cells of A549 line and human breast carcinoma cells of T47D line to 0.45 µg/ml in mink lung epithelial cells of CCL-64 line and human breast carcinoma cells of MCF-7 line.

In order to investigate toxophallin influence on cell proliferation, we performed luciferase assay with E2F reporters, which reflects activation of gene involved in cell cycle regulation. The obtained results permit one to suggest that toxophallin has no effect upon cell cycling (data not shown).

Taking into account that IC₅₀ values in cell viability test estimated by trypan blue exclusion assay (0.28 µg/ml) as well as in cell proliferation tested by [³H]-thymidine incorporation assay (0.25–0.45 µg/ml) were of the same range, it might be supposed that toxophallin possesses rather cytotoxic than antiproliferative activity.

As postulated by P. Ehrlich „Corpora non agunt nisi fixata”, the first step in the molecular mechanisms of biological action of any agent upon target cell is its binding

Table 2. Amino acid composition of the 55 kDa cytotoxic protein from *A. phalloides* fruit bodies

Composition is reported as number of residues per mole of protein based on molecular weight of 55 kDa (SDS-PAGE). The value for tryptophan was not determined (nd), but approximately calculated basing on absorption characteristics of chromatography fractions (ratio A254/A280) and mass-spectrometry data (*).

Amino acid residue	Mol %	Residues per mol of protein (calculated)
Asx	12.1	59
Thr	7.3	40
Ser	7.0	34
Glx	8.1	39
Pro	7.4	36
Gly	8.2	40
Ala	7.5	37
Cys	0.6	3
Val	7.3	36
Met	1.2	6
Ile	6.5	32
Leu	7.2	35
Tyr	3.4	17
Phe	3.9	19
His	3.4	17
Lys	5.9	29
Arg	2.9	14
Trp	ND	(10)*
Total	100	503 aa 55,153 Da

with cell surface receptor. In order to identify specific toxophallin binding proteins in plasma membrane of target cells, toxophallin was labeled with ^{125}I , and then cross-linking technique was used. Experiments using two cross-linkers, DSS and BS³ (membrane impermeable water soluble DSS analogue), did not show specific surface receptor on cells of different lines (A549, CCL-64, MCF-7) in a broad range of cross-linking agent concentrations (0.008–1.0 mM). Protein binding was a non-saturable process and was not affected by the presence of 100-fold molar excess of non-radioactive protein. Thus, toxophallin interaction with target cells does not seem to be receptor-mediated. In these cross-linking experiments, it was also found that toxophallin is capable of forming trimers in solution. In the presence of very low concentration (0.04 mM) of the cross-linker BS³, toxophallin formed a complex of molecular mass equal 160 kDa (Fig. 3) that corresponded to a trimer of 55 kDa monomers.

A possibility that toxophallin can promote cell death via apoptosis induction was studied by DNA fragmentation assay performed on different cell lines. Genomic DNA of murine leukemia L1210 cells, mink lung epithelial CCL-64 cells, and human lung carcinoma A549 cells was isolated from non-treated or toxophallin treated cells and analyzed by

electrophoresis in agarose gel. Non-treated cells did not show DNA fragmentation, while toxophallin-treated cells clearly showed DNA laddering that depended upon toxophallin doze (Fig. 4, A). Toxophallin induced DNA fragmentation at concentrations as low as 0.12 $\mu\text{g}/\text{ml}$. In case of CCL-64 and A549 cells, DNA fragmentation was not that distinct, as it was in the case of L1210 cells treated with toxophallin (Fig. 4, B). Thus, we demonstrated that target cells of different species and tissue origin undergo apoptosis under toxophallin action.

To explore the molecular mechanisms of pro-apoptotic action of toxophallin, we have studied involvement of caspases in toxophallin action. It was found that pre-treatment of L1210 cells for 2 h before toxophallin addition with 25 mM caspase inhibitor III (Boc-D-fmk), cell-permeable irreversible caspase inhibitor, did not affect toxophallin dependent apoptotic fragmentation of the DNA. In another experiment,

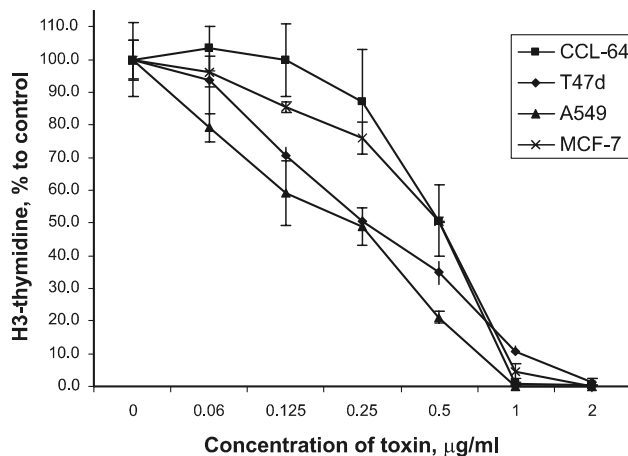


Fig. 2. Dose-dependence of toxophallin effect towards DNA synthesis in different target cells. Cell lines are noted in the box in the upper right side of the figure. Radioactivity of ^3H -thymidine-labeled trichloroacetic acid-insoluble polymers was measured in scintillation beta-counter

pre-treatment of A549 cells with Boc-D-fmk had no effect on toxophallin-induced inhibition of thymidine incorporation. Thus, apoptosis induction by toxophallin does not depend on caspase cascade pathway.

Proapoptotic action of toxophallin demonstrated in DNA-laddering assay, was proved by the results of cytomorphological study of cell nucleus using DAPI staining. The most pronounced change in cell morphology after toxophallin treatment was nuclear chromatin condensation (Fig. 5, A). For a part (3–4%) of cell population, that process (nucleus condensation) was accompanied by an appearance of apoptotic bodies which are characteristic for cells undergoing apoptosis. DNA fragmentation in the nucleus of cells treated with toxophallin, was also proved by terminal deoxynucleotidyl-transferase (TdT) mediated dUDP nick-end labeling (TUNEL assay) (Fig. 5, B). There were no TUNEL-positive cells in population of untreated cells, while the number of TUNEL-positive cells increased significantly in a concentration-dependent manner under toxophallin treatment. It correlated with the number of condensed nuclei observed at DAPI staining. Similar pattern of morphological changes in cell nuclei under toxophallin treatment was observed in various cell lines (A549, MCF-7, CCL-64).

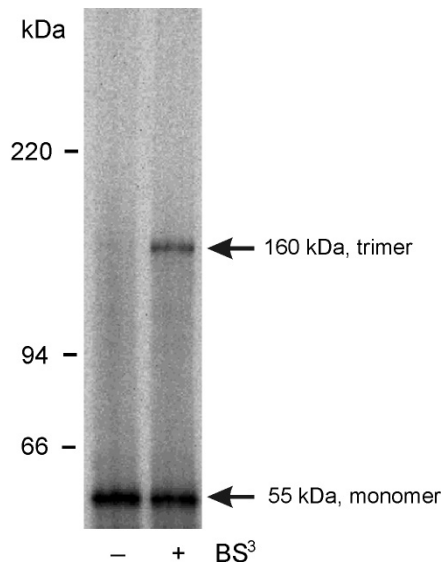


Fig. 3. Electrophoretic study of the products of cross-linking reaction using toxophallin. Toxophallin was labeled with ^{125}I and cross-linked with BS^3 (0.04 M, 15 min on ice). The products of its cross-linking with membrane proteins of L1210 cells were resolved in 5–7.5% gradient SDS-PAGE, and exposed in Fuji X 2000 PhosphorImager. Arrows show migration of toxophallin as monomer (55 kDa) and trimer (160 kDa). Migration distance of molecular mass protein markers is also shown

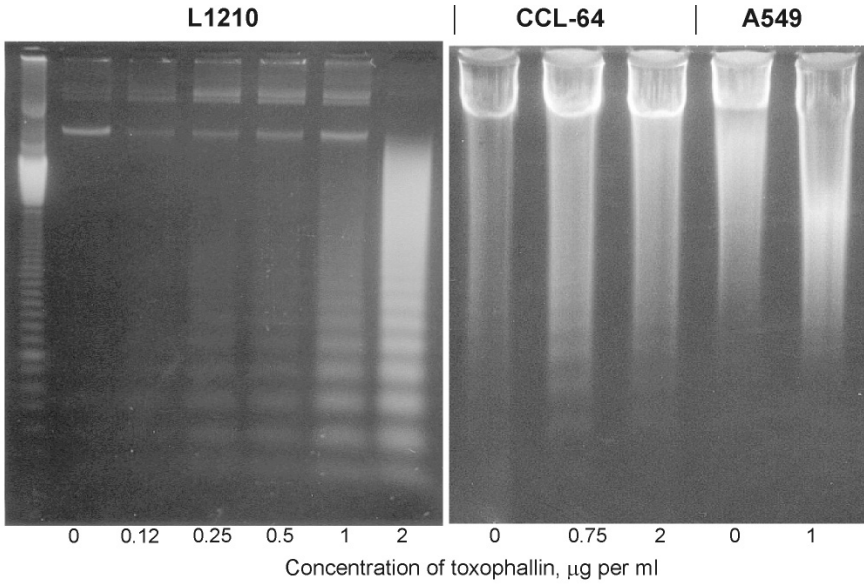


Fig. 4. Electrophoretic study of toxophallin-induced DNA fragmentation in different target cells. Cells were treated for 24 h with different doses of toxophallin. Total genomic DNA was collected and resolved in 1% agarose gel

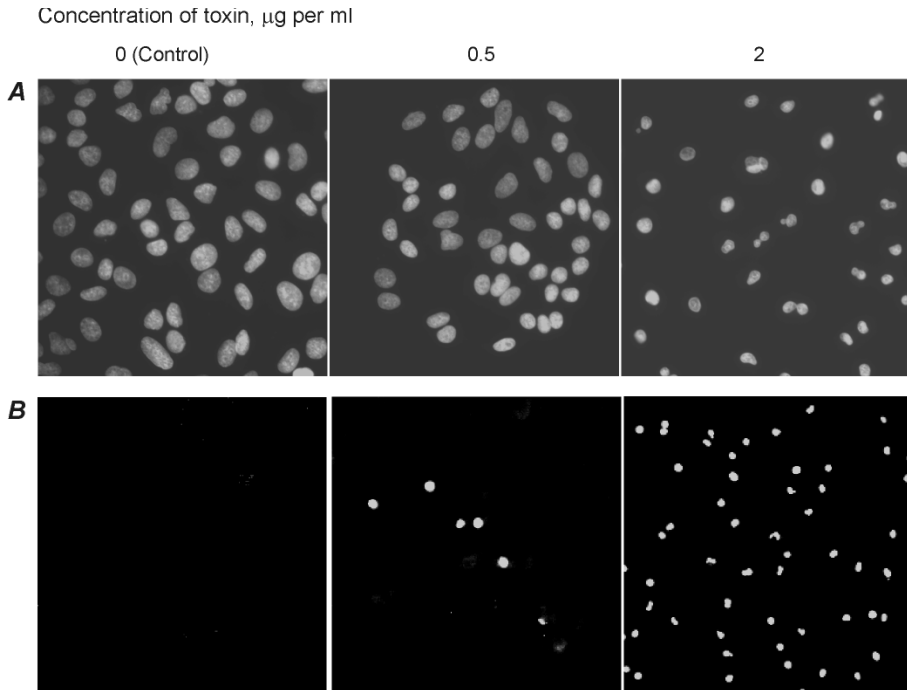
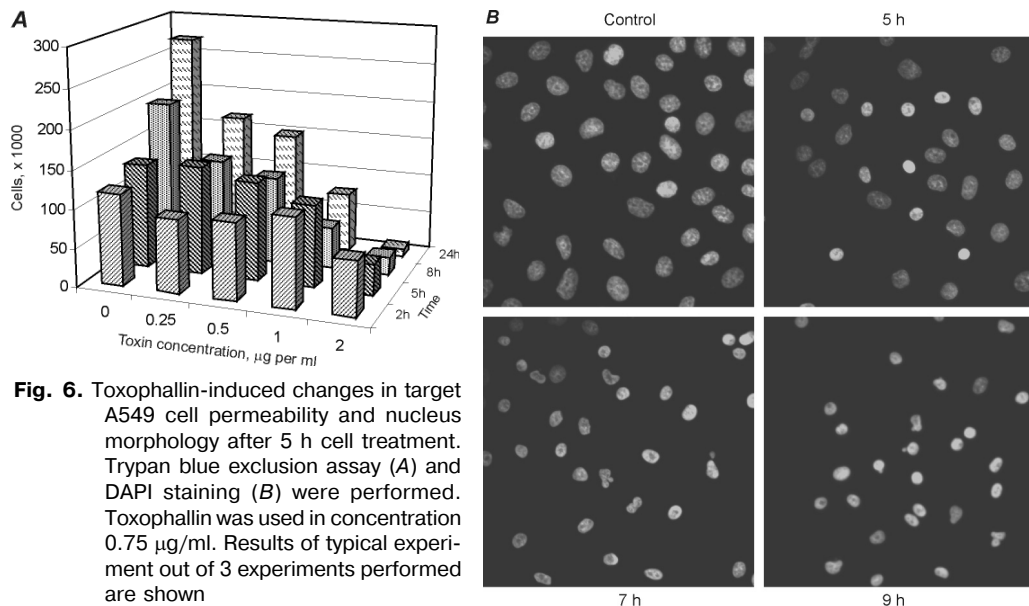


Fig. 5. Fluorescence microscopy of toxophallin-induced nucleus condensation and DNA fragmentation in A549 cells, as evaluated by chromatin staining with DAPI (A) or TUNEL (B). Representative fields of typical experiment out of 3 experiments performed are shown



Time dependence of toxophallin effect was studied by using trypan blue exclusion test and DAPI staining. The results of that study demonstrated that toxophallin-induced cell death became noticeable after 5 h action (Fig. 6, A). Nuclei condensation detected by DAPI staining after 5 h treatment with toxophallin, was the earliest toxophallin effect (Fig. 6, B) that was subsequently accompanied by apoptotic nuclei fragmentation observed in 7–9 h of toxophallin action towards target cells.

Concluding, a novel cytotoxic protein was isolated from the death cap *Amanita phalloides* and characterized in this study. According to its physico-chemical and biological characteristics, that protein does not correspond to any earlier described *Amanita phalloides* toxic substances, such as toxic cyclopeptides or phallolysin.

DISCUSSION

When studying toxic proteins isolated from fruit bodies of the death cap *Amanita phalloides* by using approaches of both biochemistry and cell biology, we detected a novel toxic protein differing from earlier described toxic protein (phallolysin) of that mushroom species. In the present study, an improved procedure for purification of that protein was developed and detailed investigation of its physico-chemical properties and biological activity was conducted.

It was found that this novel toxic protein differs distinctly from phallolysin which was isolated and characterized by Seeger et al. [16–18] and Faulstich et al. [4, 5]. Some of those characteristics are presented in Table 3.

Both proteins differ substantially in their biological activity. Phallolysin is highly toxic in animals reaching a lethal dose 40 µg/kg in rabbits [4]. In our in experiments based on using cultured L1210 cells, the cytotoxic dose ID_{50} equaled 5 µg/ml. It was found that toxophallin exhibited high toxicity towards mammalian cells, for example, IC_{50} was equal 0.25 µg/ml in cells of L1210, A549, and T47D lines, and it was 0.45 µg/ml in cells of CCL-64 and MCF7 lines. No hemolytic activity was

detected in toxophallin preparations, while the hemolytic activity of phallolysin achieved 24,000 units/mg [5].

It should be noted that toxophallin mRNA (2.1 kb) is expressed mainly in the stem and to a less extent in the cap of *Amanita phalloides* fruit bodies. No homologous toxophallin mRNA was found in fruit bodies of other species of *Amanita* genus such as *Amanita rubescens* or *Amanita spissa*, and in *Phallus impudicus*, as revealed by Northern-blot analysis using RT-PCR fragment of cloned toxophallin cDNA, as a probe for hybridization reaction (these our data are not presented in this paper). Those results suggest a non-ubiquitous expression of toxophallin in *Amanita phalloides* related mushroom species.

Table 3. Comparison of physico-chemical properties of toxophallin and phallolysin

Properties	Toxophallin	Phallolysin
Molecular mass	57 kDa	34 kDa
pI	5.7	7.0–8.1 (three isoforms)
Characteristic features of amino acid composition	Rich in proline, also contains cysteine and methionine	Rich in serine, threonine, glycine, and lysine, and lacks cysteine and methionine

Cross-linking receptor study did not reveal specific receptor molecules for this protein on the surface of target cells. It was found that cytotoxic effects develop relatively slowly, since the first signs of cell damage were observed only in 6 hours of cell treatment. Target cells were undergoing apoptosis at toxophallin treatment, and cell death did not depend upon activation of caspase cascade. The most pronounced destructive changes were observed in the cell nucleus, namely condensation of nuclear chromatin and DNA fragmentation. These processes are characteristic for cell damage caused by ionizing radiation and they are mediated by generation of reactive oxygen species (ROS). Thus, it might be suggested that toxophallin induces cell damage indirectly via generation of free radicals and oxidant agents which can trigger cell damage and apoptosis by caspase-independent pathway.

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