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Effects of Serine Enzyme Extracted from Earthworm Lumbricus Terrestris in the Expression of Apoptosis-Related Protein on the MCF-7 Cell Line

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ABSTRACT

Background and Objective: Cancer is the leading cause of death worldwide, and breast cancer is the most common type of cancer in women worldwide. Earthworms have been used in medicine to cure a variety of diseases because their body extracts have a variety of bioactive substances (such as proteins and peptides). Serine enzyme is an enzyme purified from the earthworm with a significant antitumor activity against human cancer cell. The aim of the study is to determine the mechanisms by which serine proteases induce apoptosis and to examine the expression of a number of apoptosis-associated proteins in treated and untreated (MCF-7) cell line using microarray.

Methods: In this laboratory study, healthy and fully mature earthworms were collected. After seven days of growing in humus-enriched soil with intermittent water sprays in a controlled laboratory setting. Lumbericas terrestris serine protease was added to $1x10^5$ cells/mL of MCF-7 cells after they were seeded, and the treatment lasted for 24 hours at IC50 concentration. Untreated cells were employed as a negative control. Following a 24-hour treatment period, MCF-treated cells were collected and spun down for five minutes at 2500 rpm and 4°C. Serine enzyme was partially purified from earthworms Lumbricus terrestris and the MCF-7 cell line was treated with serine enzyme.

Findings: The results showed that treatment of MCF-7 cells with serine protease led to changes in most apoptosis-related proteins. Treatment of MCF-7 cells with serine protease resulted in downregulation of cIAP-2 (632±7.2 & 275±4.8), Livin (220±4.7 & 95±2.1), XIAP, Survivin, BCLw, BAD, BAX, BID, BIM, cytoC and P53 with an increased expression of caspase 3 (83±1.5 & 493±4.9), caspase 8 (285±3.33 &610±7.65) and SMAC compared to untreated cells.

Conclusion: The results of the study showed that serine protease has the ability to facilitate perforation of mitochondrial membrane as well as caspase 3 cleavage in apoptotic cell death.

Keywords: *Serine, Earthworm, Mcf-7, Apoptosis Protein, Microarray.*

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Introduction

Cancer is a leading cause of death globally. One in five individuals worldwide develop cancer during their lifetime. The most frequent cancers are breast, lung and colon (1). Breast cancer is a type of cancer which usually starts inside the breast tissue and spreads to other parts of the body (2). Approximately 2.3 million new cases of breast cancer are reported each year, making it one of the most common diseases that impact millions of people globally. It is also the leading cause of death for cancer-afflicted women in 95% of countries (3). A number of environmental agents, genetic, and lifestyle factors may cause the breast cancer. Obesity, alcohol consumption, lack of physical activity, and overweight has been linked to 21% of all breast cancer deaths worldwide (4).

A variety of medical procedures, including mammograms, breast magnetic resonance imaging (MRI), breast ultrasounds, breast inspections performed by a physician, and the removal of a sample of breast cells for testing (biopsy), are recommended to detect breast cancer (5, 6). Since 1340 AD, earthworms have also been used in medicine to cure a variety of diseases. Their body contains a variety of bioactive substances (such as proteins and peptides) that protect these worms. Proteases, metabolites, metal-binding proteins, active proteins (such as lysozyme, lysenin, and eiseniapore), (lumbricusin, lumbricine, and lumbricin I), and organic acids and compounds are among the bioactive substances that have therapeutic potential (7). Shafi et al found that the earthworm, Lumbricus terrestris, extract have bioactive effect on both breast and prostate cancer (8).

Serine proteases are enzymes produced by the digestive system of earthworms (9, 10). The first person who studied an enzyme derived from earthworms that broke down fibrin was Fredericq, who discovered a protease from their fluid in the 19th century (11). The digestive tracts of earthworms produce the enzymes known as earthworm serine protease (ESP). Numerous therapeutic benefits of ESP have been documented and its fibrinolytic activity has been well investigated. ESP isoforms represent complex enzyme with molecular weights ranging from 14 to 33 kDa (9). The researchers in a study extracted serine enzyme and found that serine enzyme extract from Lumbricus terrestris have antitumor activity against A549 cancer cell line (12). The present work aims to determine the mechanisms for the induction of apoptosis by serine proteases and the expression of a number of apoptosis-associated proteins in treated and untreated MCF-7 cells using microarray.

Methods

This laboratory study was approved by the Ethics Committee of the Faculty of Sciences of Mustansiriyah University with the code BCSMU/0222/00025Z.

Collection and processing of earthworms: Healthy and fully mature earthworms were collected from Baghdad, Iraq, after seven days of growing in humus-enriched soil with intermittent water sprays in a controlled laboratory setting. Autolysis began when the mature earthworms were repeatedly washed in sterile distilled water. To induce autolysis, 20 mmol/L of phosphate buffer (pH 7.5) with sodium azide 0.02% as a bacteriostatic was heated to 60 degrees Celsius for three hours. Worms were stored in the refrigerator at 15°C for a week to induce autolysis. Afterwards, the worms were centrifuged at high speed for 30 minutes (4°C, 16 000 r/min). The supernatant was stored in sterile tubes away from any potential contamination. The supernatant was filtered via Whatman filter paper to remove any remaining tissue fragments before being purified (13). Purification of serine protease isolating protease from earthworms was done using the methods described by some previous studies (14, 15). Ammonium sulfate was used to precipitate the total protein from crude, and it was subsequently reconstituted in phosphate buffer saline (pH

7.5). A dialyzing membrane with 20 kDa molecular weight cutoff cup was used to concentrate the protein and remove the salt from it. The next step was the separation of protein using ion exchange chromatography (IEC) on the DEAE-cellulose column. Before the column was prepared, the resin was charged with 30 minutes of HCl, 30 minutes with NaOH, and 30 minutes with distilled water. Approximately, 90 fractions were obtained, and their protein activity and content were examined. The final step was six fractions with higher protein content subjected to Sephadex G50 beads for column packing under gravity. Packing and equilibration of the column was done using PBS (pH 7.5). 5ml of DEAE-cellulose and purified and pooled protein faction was loaded into a column and eluted with excess PBS (7.5). The fractions were collected and profiled for protein concentration and activity analysis.

Morphological Identification: Morphological identification of earthworms depends on the external features (16). Earthworm identification was confirmed by Dr. Nebrass Faleh Chachain, Biology department, college of science, Mustansiriyah Univesity. Earthworms were washed with water to remove any mud and anaesthetized in diluted ethanol 10% then ethanol 30% for 10 minutes and examined under the dissecting 40x microscope lens. The external feature for identification includes: Body color, Body length and number of segments, Setae arrangement, Shape and segment of clitellam, Location and shape of tubercula pubertatis (TP), Location of male pores, and Glandular tumescences.

Cell culture: Michigan Cancer Foundation-7 (MCF-7) was suspended in RPMI1640 medium and was allowed to proliferate for 24 h at 37°C supplemented with 5% carbon dioxide. When the cultured cells reached a confluency of about 90%, the growth medium was removed and the attached cells were washed with sterile phosphate buffer saline three times. To collect the attached cells, 2-3 milliliters of trypsin- EDTA solution was added to the flask. Then, the flask was turned over and shaken gently to cover all the attached cells. To top off the detachment process, the flask was incubated at 37°C. Trypsin activity was stopped by adding complete RPMI1640 medium. The cell suspension was distributed to other flasks containing fresh complete RPMI1640 medium. The flasks were incubated at 37°C using 5% carbon dioxide.

Microarray: Human Apoptotic Proteins Array: As instructed by the manufacturer, the role of apoptotic proteins in apoptosis was determined by utilizing an apoptosis array and the Human Apoptosis Antibody Array kit (RayBiotech, GA, USA). Briefly, Lumbericas terrestris serine protease was added to 1x105 cells/mL of MCF-7 cells after they were seeded, and the treatment lasted for 24 hours at an IC50 concentration. Untreated cells were employed as a negative control. Following a 24-hour treatment period, MCF-treated cells were collected and spun down for five minutes at 2500 rpm and 4°C. Ice-cold PBS was used twice to wash the cells. After five minutes of centrifugation at 2500 rpm and 4°C, the supernatant was disposed of. Cell proteins were extracted and approximately 500 µg of proteins from each sample were incubated with a human apoptosis array overnight. The Odyssey Fc Imaging System (LI-COR, USA) was used to scan the membrane for chemiluminescence detections (RayBiotech, GA, USA).

Statistical analysis: Data are shown as mean and standard deviation, and statistical analysis was done with Graph Pad Prism 6 (Graph Pad Software, USA) using a t-test. Differences were considered as being significant at $p \leq 0.05$.

Results

Result indicated that treating MCF-7 cells with a serine protease cause alteration in most apoptotic related proteins. Treatment of MCF-7 cells with the Serine protease resulted in downregulation of cIAP-2 (632±7.2 & 275±4.8), Livin (220±4.7 & 95±2.1), XIAP, Survivin, BCLw, BAD, BAX, BID, BIM, cytoC and P53 with an increase expression of caspase 3 (83±1.5 & 493±4.9), caspase 8 (285±3.33 &610±7.65) and SMAC versus untreated cells. The data for the expression of apoptosis-associated proteins in untreated

MCF-7 and MCF-7 treated with serine protease are shown in Table 1. The treatment of MCF 7 cells with a serine protease for 24 h displayed modifications in apoptotic related proteins as showed in Table 1 and Figure 1. Table 1 demonstrates the levels of protein measured by fold change of the inhibitors of apoptosis proteins. The expression of proteins (BCLw, XIAP, Livin, Survivin and cIAP-2) showed significant down-regulation compared to untreated cells (folding change-0.43; p-value 0.001: folding change-2; p-value 0.001; folding change-1.2; p-value 0.001; folding change-0.5; p-value 0.001; folding change-1.2; p-value 0.001, respectively) accompanied with increased expression of mitochondria protein SMAC (folding change 1.13; p-value 0.001) as well as more than two fold increase in level of caspase (caspase 3 and caspase 8) (folding change 2.5; p-value 0.001, folding change 1.09; p-value 0.001, respectively), indicating clear signs of apoptotic induction. On the contrary, a significant decrease in level of proapoptotic protein including BAD (folding change -0.45; p-value 0.001), BAX (folding change-0.47; p-value 0.001), BID (folding change -3.47; p-value 0.0001) and BIM (folding change -0.15; p-value 0.05) combined with a significant reduction in level of cytoC (folding change -1; p-value 0.001) together with nonsignificant decrease in the level of P53 protein (folding change -0.07) was seen in cells treated with serine protease. The current results found that the treated MCF-7 with the serine protease induce downregulation of all inhibitors of apoptosis proteins along with an increased expression of caspase 3 and caspase 8, with increased level of SMAC and more varied impact on the caspase 8 activity.

Table 1. The mean±SD and folding change of several apoptosis-related proteins in MCF-7 cell line treated with serine protease

Member of apoptosis-related proteins	Before Mean±SD	After Mean±SD	p-value	Folding change
Member of the inhibitor apoptosis (IAP)				
cIAP-2	632±7.2	275±4.8	0.001	-1.2
Livin	220±4.7	95±2.1	0.001	-1.2
XIAP	194±3.66	54±2.1	0.001	-2
Survivin	437±6.1	296±3.7	0.001	-0.5
Mitochondrial proteins				
SMAC	420±4.9	923±11.8	0.001	1.13
cytoC	418±4.6	223±6.4	0.001	-1
Caspase				
Caspase3	83±1.5	493±4.9	0.001	2.5
Caspase8	285±3.33	610±7.65	0.001	1.09
Pro apoptotic				
BAD	853±12.5	629±6.4	0.001	-0.45
BAx	507±6.2	366±4.9	0.001	-0.47
BID	160±2.6	15±1.3	0.001	-3.47
BIM	444±6.1	408±4.8	0.05	-0.15
Antiapoptotic				
BCL-2	185±5.1	265±3.9	0.001	0.5
BCL-W	279±4.2	209±3.11	0.01	-0.43
Suppressor protein				
P53	266±3.5	253±4.1	ns	-0.07

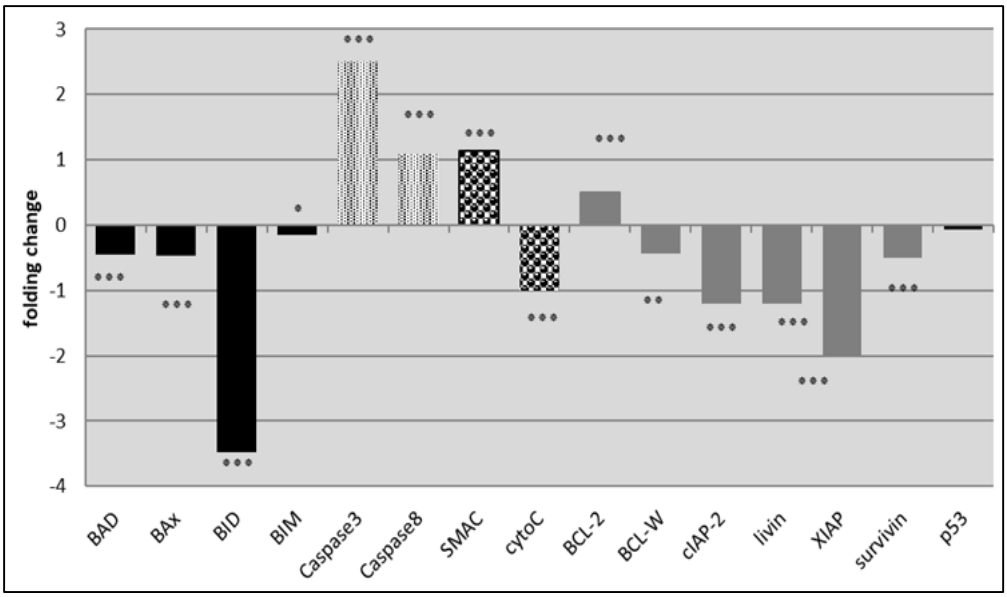

Figure 1. Results of analyzing the protein microarray involved in apoptosis in MCF-7 cells after treatment with serine protease enzyme; Data are expressed as folding change. The statistical significance was determined comparison with the untreated cells.

Discussion

These results provide testimony in support of the hypothesis that serine protease have the ability to facilitate perforation of mitochondrial membrane and casp-3 cleavage in apoptosis. These data denote that the serine protease induce apoptosis through intrinsic pathway by matrix metalloproteinase, depolarizing and then activating downstream caspases. Previous studies demonstrate that serine protease acts in an apoptosis-like fashion via a mechanism that damages the mitochondrial membrane (17). The serine proteases initiate mitochondrial membrane permeability in contrast to the serine protease Omi/HtrA2, which is liberated from mitochondria at a later stage (18).

In conclusion, MCF-7 cancer cells treated with serine protease result in activation or inhibition of many proteins that have essential role on the future fate of the cell. Serine enzyme regulates cell death pathway through different mechanisms, the first one being involvement in the cleavage and activation of different proteins involved in apoptosis process such as caspases. The, second mechanism includes cleave nuclear matrix antigen and poly (ADP-ribose) polymerase, which play a role in DNA damage during cell death; the third mechanism involves pro-apoptotic protein BID and convert it to truncated Bid (tBid). They translocate to the mitochondria and then release apoptotic factors via increased permeability of mitochondrial outer membrane and activation of downstream apoptotic cascades. Serine enzyme also plays a role in necrosis, autophagy, pyroptosis, and other processes of cell death. By stimulating the release of pro-inflammatory cytokines, they also play a significant role in inflammatory processes and immune system function.

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