Influence of various levels of L-asparaginase II purification on the cytotoxicity, DNA level, and apoptosis in Hep-2 cells

تأثيرالمستويات المختلفة من التنقية لأنزيم L–asparaginase II في السمية الخلوية ، مستوى إلـ DNA وعملية الموت المبرمج للخلايا السرطانية Hep–2

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Abstract

The genetic effects of several concentrations of L–Asparaginase II (ASNase II), produced by *Proteus vulgaris* strain Pv.U92, at various levels of purification (ultrasonication, precipitation, ion-exchange chromatography and gel filtration chromatography) on cancer cells line of Hep–2 were studied. This bacterial enzyme with concentration 4 U/ml at gel filtration level was revealed a putative cytotoxicity against cancer cells in comparison with other concentrations and steps of purification were used in this work. Moreover, 4 U/ml of ASNase II at gel purification level has a distinguished role on arrest cancer cells division of Hep–2; it was reduced the content of DNA at each phase of cancer cell cycle particularly at G_2/M phase, the level of DNA was 3%. On the other hand, the partial purified enzyme, L–ASNase II, was induced apoptosis by both levels of purification ion–exchange and gel filtration, the apoptotic fractionation was 0.86 and 0.7 respectively.

المستخلص

درست التأثيرات الوراثية لتراكيز مختلفة من أنزيم L–Asparaginase II (ASNase II) المنتج من السلالة البكترية *Proteus vulgaris* PVU92 وبمستويات مختلفة من التنقية (الامواج فوق الصوتية ، الترسيب ، كروماتوكرافيا التبادل الايوني ، كروماتوغرافيا الترشيح بالهلام) في الخلايا السرطانية من نوع Hep–2 . أظهر الستخلص البكتري بتركيز4 وحدة / مل وبمرحلة الترشيح الهلامي فعالية سمية في الخلايا السرطانية مقارنة مع بقية التراكيز ومراحل التنقية المستعملة في هذه الدراسة . فضلا عن ذلك كان للانزيم البكتري بنفس التركيز وبمرحلة الترشيح بالهلام دورا مميزا في أيقاف أنقسام الخلايا السرطانية Hep–2 حيث تمكن من خفض محتوى الدنا في كل طور من أطوارالأنقسام بحيث بلغ محتوى الدنا في طور G_2/M 3% . ومن جانب اخر فقد تمكن الانزيم ASNase II المنقى جزئيا والمستعمل بمرحلتي التبادل الايوني و الترشيح بالهلام من تحفيز موت الخلايا السرطانية وقد بلغت النسبة المئوية لأجزاء الخلايا المنتحرة 0.86 , 0.7 على التوالي .

Introduction

L-asparaginase (ASNase) is L-asparagine amido hydrolase enzyme, the systematic code is E.C. 3.5.1.1, it belongs to an amidase group that produces aspartic acid and ammonia by asparagines hydrolysis [1].

L-asparaginase II(ASNase) presents in almost Enterobacteriaceae in two types: ASNase I and ASNase II. ASNase I have low affinity and located at cytoplasm, while

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ASNase II has high affinity and located at periplasm. Only ASNase II has anti-lymphoma activity [2].

Normal human cells are able to make L-asparagine they need internally by L-asparagine synthetase (ASNase), in contrast neoplastic cells lacking adequate level of ASNase because it's gene is highly methylated [3], therefore tumor cells require huge amounts of L-asparagine to keep up their rapid malignant growth; it means they use both L-asparagine from diet as well what they can make themselves to satisfy their large L-asparagine demanded [4].

In present study, an attempt to evaluate the cytotoxicity and apoptosis of ASNase II at different steps of purification against cancer cell line Hep-2 was done.

Materials and Methods

L-Asparaginase synthetase II (ASNase II)

ASNase II at various steps of purification (ultrsonication, precipitation, DEAE-cellulose exchange chromatography, and sephacryl S-300) was produced by strain of *Proteus vulgaiis* Pv.U92 that kindly provided by [5].

Cell culture

Human epidermoid larynx carcinoma (Hep-2) cell line at passage 323, kindly provided by Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), was cultured in RPMI medium 1640 containing 2mM L-glutamine, and supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured at 37ºC in a humidified incubator with 5% CO_2 [6].

Examination of cytotoxicity of ASNase II

MTT [3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide)] assay was conducted as previously described [7]. In briefly, $2x10^5$ cells per well were seeded onto 96-well culture plates in 200µl RPMI 1640 medium and incubated for 24 h. After incubation period, the cells were washed with same medium and then replaced with fresh medium containing different concentrations of ASNase II (0.25, 0.5, 1, 2, 4) U/ml, each concentration had three repeated wells. The plates were incubated at 37ºC for (3, 24, 48) hr. The non-treated cells were represented the negative control. After incubation period, 10µl of 5mg/ml MTT solution was added to the culture and incubated for 4h at 37ºC. Subsequently, medium was sucked out and 100µl of dimethylsulfoxide (DMSO) was added to dissolve the violet crystal and the MTT formazan was spectrophotometrically measured at 570 nm. The percentage of inhibition rate was estimated [8].

Cell cycle analysis of treated Hep–2

Approximately 1.5×10^6 cell/ml of Hep-2 was treated with 4U/ml of ASNase II at different levels of purification and incubated for 72h at 37ºC. Subsequently, cells were collected by centrifugation (500xg for 5 min.) and treated with 10µM of BrdU for 30min. at 37ºC; cells were washed with phosphate buffer solution (PBS) and re-suspended with 1 ml of Borax buffer. The step of washing was repeated twice. Thereafter, cells were re-suspended in 200 µl of PBS and labeled with 4µl of goat-anti-mouse FITC-conjugated antibody, incubated for 30 min. at 4ºC in the dark. Cells

were resuspended with 200 µl of Propidium Iodide (PI) and analyzed with flow cytometer at 488 nm Argon laser [9].

Measurement of apoptotic cell fractionation

A suspension of 1.5×10^6 cell/ml of Hep-2 was plated in 96-well microplate, treated with 4U/ml of ASNase II at various levels of purification and then incubated at 37ºC for 3h. After incubation, the adherent cells were treated with trypsin to remove them from the plate surface; cells (the floating and detached cells) were collected by centrifugation at 1500 rpm for 10 min. and washed with PBS. The pellets were re-suspended in 4 ml of 3.7% paraformaldehyde and left for 20 min. Then, the cells were centrifuged at 1500rpm for 10 min., washed twice with PBS, re-suspended in 20 ml PBS, deposited it on polylysine-coated cover slips, and left to adhere on coverslips for 30 min at 25ºC followed by washing twice with PBS. Thereafter, 0.1% Triton X–100 was gently added, left for 5 min. at 25ºC, then rinsed three times with PBS. The cover slips were incubated with Hoechst 33258(1 mg/ml) for 30 min at 37ºC.The cover slips were rinsed with PBS and amount on slides with glycerol–PBS (1:1). The apoptotic cell fraction was calculated by taking the ratio of floating cells: total cell number [10].

Statistical analysis

Experimental data were analyzed using ANOVA at probability level $P< 0.05$.

Results and Discussion

Cytotoxicity of ASNase II at different concentrations and various levels of purification on cancer cell line Hep-2:

At ultrasonication step, all concentrations of ANSase II were decreased the displayed effective inhibition rate (61.4%) after treatment for 24 h. Figure (1).

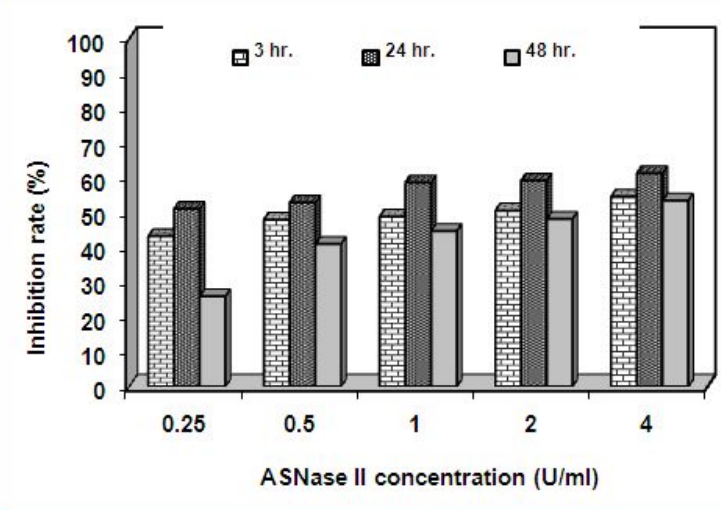

Fig (1): Cytotoxic effect of ASNase II at ultrasonication level of purification on Hep-2cell

While, the cytotoxic activity of ASNase II at precipitation step was increased gradually with increasing concentrations Figure (2). Remarkably, the concentration 4U/ml of ASNase II was a potent inhibitor against cancer cells after 24 h incubation, the inhibition rate was 54.7%. Whereas, ASNase II at ion exchange level (using

DEAE–cellulose resin) of purification was significantly (P<0.05) exhibited antiproliferative activity against Hep–2 cells at concentration 2 and 4 U/ml after 3h; the inhibition rate was (56 , 57.5)%, respectively Figure (3).

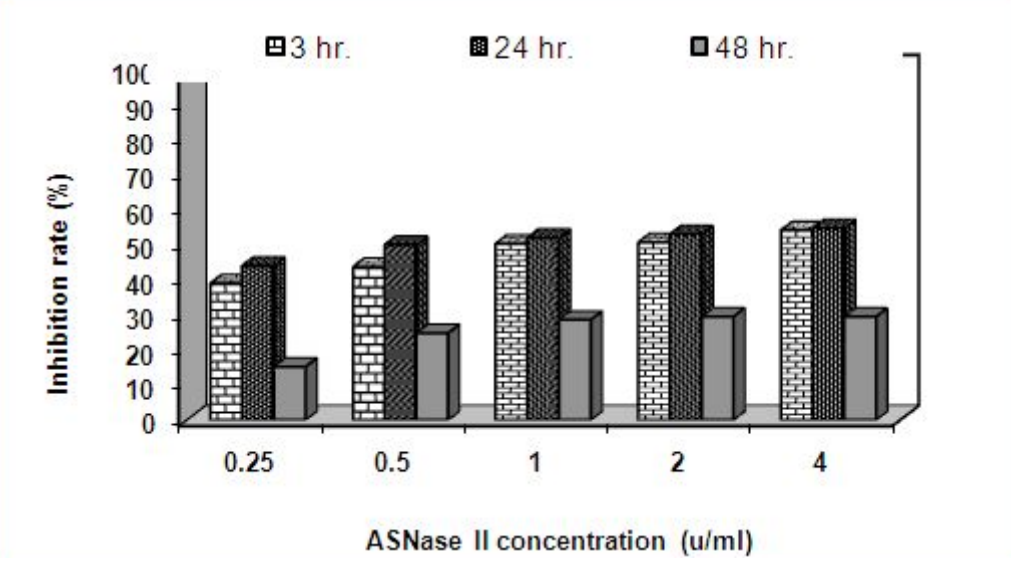

Fig (2): Cytotoxic effect of ASNase II at precipitation level of purification on Hep-2cells.

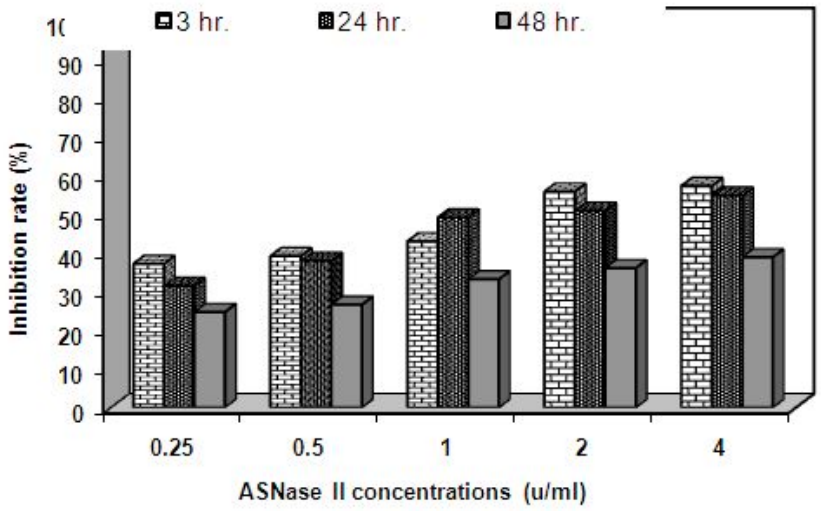

Fig (3): Cytotoxic effect of ASNase II at ion-exchange (DEAE-cellulose) level of purification on Hep-2 cells

On the other hand, ASNase II at gel filtration (using Sephacryl S-300) showed a significant (P<0.05) inhibitory activity at 4U/ml after 3h from treatment; the inhibition rate was 59.8% Figure (4).

Although the inhibitory activity of ASNase II was increased after 48 h through increasing the concentrations of ASNase II at all purification levels used in this experiment, non- significant (P<0.05) cytotoxicity were seen in comparison with other levels of purification Figure (1, 2, 3, 4).

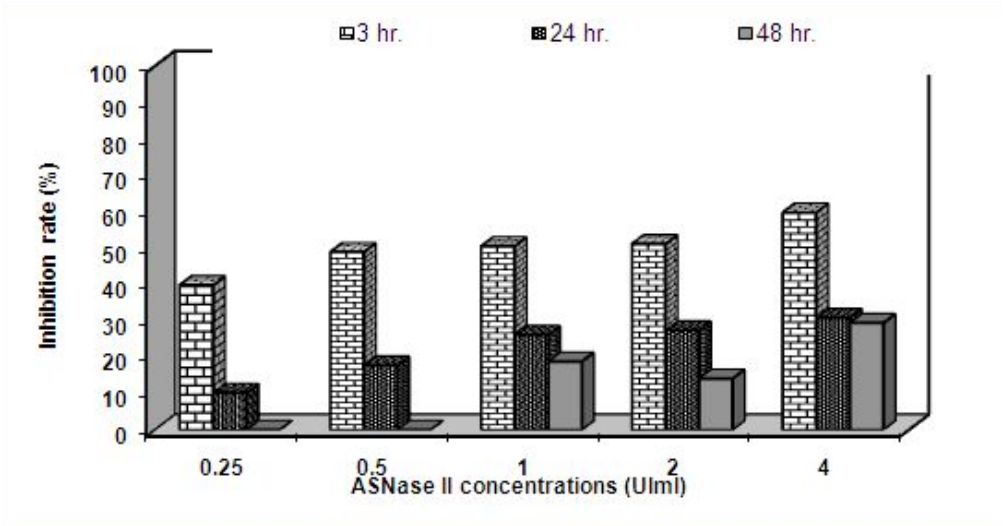

Fig (4): Cytotoxic effect of ASNase II at gel filtration (Sephacryl S-300) level of purification on Hep-2 cells.

Effect of ASNase II at various levels of purification on DNA content in the cell cycle of Hep–2

Table (1) demonstrated the inhibitory effect of 4U/ml of ASNase II, at several levels of purification, on cell cycle and DNA content of Hep-2 cells. Remarkably, DNA content in Hep-2 cells treated with ASNase II at gel filtration step was significantly (P<0.05) low at G_0/G_1, S, and G_2/M phases (90, 7, 3) %, respectively. The next level of purification that influence DNA content in Hep-2 cell cycle was ion exchange; the DNA content was (94, 1, 5)% at G_0/G_1, S, and G_2/M, respectively.

In regard to the rest purification levels (Ultrasonication and precipitation), they showed less effect in cell cycle than above levels.

Table (1): Percentage of DNA level at each phase of cell cycle of Hep-2 treated with partial purified ASNase II .

Purification steps of ASNase II	Percentage of DNA content at different phases of Hep-2 cell cycle		
	G0/G1	S	G2/M
Ultrasonication	67	20	13
Precipitation	75	15	10
Ion-exchange using DEAE-cellulose	94	1	5
Gel filtration using Sephacryl S-300	90	7	3

Effect of ASNase II at various purification levels on apoptosis of Hep–2 cell

Figure (6) indicated that Hep–2 cells was very sensitive to ASNase II especially at gel filtration level of purification, the fraction of apoptotic cells was significantly high; it performed 0.86. While ANSase II at ion-exchange purification level was achieved a low apoptotic cell fractionation 0.7 with non-significant differences (P<0.05) in comparison with above treatment. Whereas ultrsonication and precipitation steps of purification were revealed low efficiency in apoptosis; the fractionation of apoptotic cell was 0.5 and 0.4, respectively.

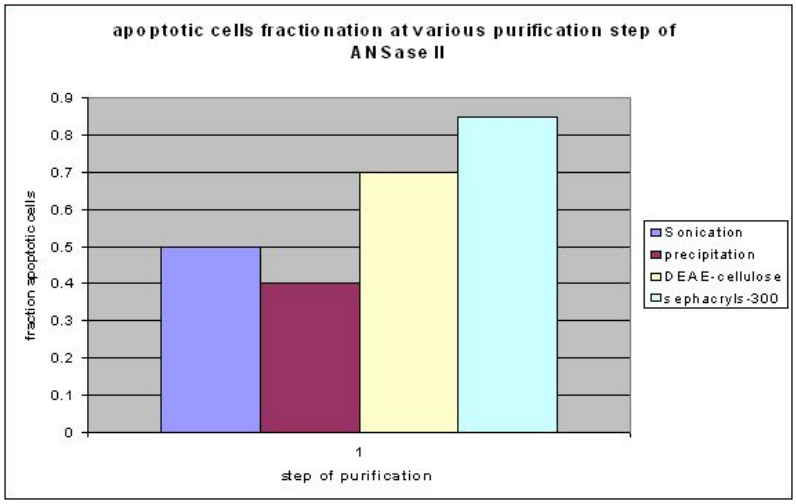

Fig (5): Apoptotic cell fractionation at various purification level of ASNase II.

Discussion

Bacterial ASNase II is an antitumor neoplastic drug used in the treatment of cancer such as lymphoblastic leukemia [11], melanosarcoma cancer [12], and lymphosarcoma [13]. In this study ASNase II showed variation in its activity against cancer cells of Hep-2 according to the level of purification. In regard to the cytotoxicity it has been indicated that ASNase II at ultrasonication level had a potent antiproliferative activity against cancer cells in comparison with other level, this may return to the presence of other compound in the extract which supports ANSase II activity. This compound is lipopolysaccharide (LPS) which its antitumor activity was proved [14]. When the cytotoxicity of ANSase II was compared with other level of purification (precipitation, ion-exchange chromatography, and gel filtration) it was appeared that it decreased with non- significance (P<0.05). This may due to the elimination of compounds found in the extract [15].

On the other hand, the sequence of events leading to arrest cell cycle and death of Hep-2 cells at G_2/M phase by ASNase II is lacked. The arrest of cell cycle may retain to the decrease in the expression of key G_2/M–regulating proteins, including cyclin B1, cell division cycle 25B (Cdc 25B), and Cdc 25C [16].Whereas, the ability of ASNase II, at several levels of purification, to induce apoptosis may due to up–regulation of Bax, down-regulation of Bcl–2, and activation of caspases–3,–9, and –8. Activation of caspases leads to cleavage and inactivate of cellular proteins which in turn activate mitochondrial pathway involving release of cytochrome c, thereafter caused DNA fragmentation [17].

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