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Research Article

Production, Purification and Characterization of a novel L-asparaginase from *Acinetobacter baumannii* with anticancerous activity

Sahira Nsayef Muslim[Á*]

[Á]Department of Biology, College of Science, Mustansiryia University

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Abstract

L-asparaginase is an enzyme that catalyzes the conversion of L-asparagine to L-aspartate and ammonia. The important application of the L-asparaginase enzyme in using it as chemotherapeutic for its anticarcinogenic potential .In the present study a novel strain, AcinetobacterbaumanniiR7 was explored for the production of extra- cellular L-asparaginase .This enzyme was purified by single chromatography step to homogenicity with a recovery yield of 77% and 92.9 fold of purification by using isopropanol (1:2) and CM-Sephadex C-50 chromatography. The enzyme appeared as a single protein band on SDS-PAGE gel with a molecular mass corresponding to 160 kDa. The Purified enzyme does not possess any glutaminase activity. The $K_m$was calculated as 22 mg/ml and V_{max}as 625 U/mg of protein using L-asparagine as substrate. L-asparaginase purified from AcinetobacterbaumanniiR7 at a concentration of 0.2 mg/ml showed better toxicity on OAW-42 cell line (59 and 51 % survival) for 24 and 48 h, respectively, in comparison with controls, and this result led to increase the benefit by using the enzyme for the treatment of human ovarian cancer.

Keywords:*L-asparaginase, AcinetobacterbaumanniiR7, anticancerous activity*

Introduction

Rhizosphere is a rich source of various microorganisms that can benefit plant growth and survival. Among those *Bacillus, Enterobacter, Acinetobacter, Alcalogenes, Arthrobacter, Flavobacterium, Rhizobium, Erwinia and Serratia*(1).

Acinetobacter baumannii causes opportunistic infections because of limited number of virulence factors and are thus considered as low grade pathogen (2). *Acinetobacterbaumannii* are able to survive on moist and dry surfaces, are found on fruits and vegetables and on the health human skin (3), so that the environment, soil, water and animals are the natural habitats for it (4) .Non specific nutritional requirements, resistance to desiccation and the ability to form biofilm permit *Acinetobacter baumannii* to grow in various environment, and therefore initial contact with the pathogen preceding infection can be made in a variety of ways (5, 4).

L-asparaginase enzymes (L-asparaginase amidohydrolase, EC 3.5.1.1) catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia. Since L-asparagine is very essential amino acid for the growth of tumor cells whereas the growth of normal cell doesn't of its requirement (6, 7). In the presence of L-asparaginase, the tumor cells deprived of an important growth factor and they may failure to survive. Thus this enzyme can be used as a chemotherapeutic agent for the treatment of ALL (mainly in children) as a potent antitumor or antileukematic drug (6). L-asparaginase is an important enzyme as therapeutic agents used in the treatment of Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticlesarcoma and melanosarcoma (6, 8).

L-asparaginase is found in many tissue, bacteria, plant and in the serum of certain rodents, not of man. The microbial source is very common for L-asparaginase, because they can be easily cultured and extraction purification of L-asparaginase from them is also convenient, facilitating for the industrial scale production (6).

The most commonly used microorganisms to produce L-asparaginase are *Erwinia carotovora, E. coli, Pseudomonas aeroginosa, Corynebacterium glutamicum and Bacillus* sp (7), therefore ; there is a continuing need to screen newer organisms in order to obtain strain capable of producing new and high yield of L-asparaginase .Because of there is not any report about L-asparaginase production and purification by *Acinetobacter baumannii* , In the present study, an attempt has been made to investigate the production of L-asparaginase by *Acinetobacter baumannii* besides to purify and characterize this enzyme and studying its effect on cancerous OAW-Y2 cell line also has been assessed.

Material and Methods

Isolation of bacteria

*Corresponding author: **Sahira Nsayef Muslim**

Forty four soil samples were collected from the rhizosphere area of cucumber and lettuce plants that cultured in a field in Baghdad city. One gram of each sample was suspended in 10 ml of distilled water and shaken for 15 min. Later, 0.1 ml of this suspension was spreaded on the blood agar and MacConkey agar. The plates were incubated at 30º C for 24 h(9).

Morphological Identification

The pure culture isolates were identified to species level by doing some biochemical characterization such as oxidase and catalase tests, motility test, and growth at 14,37 and 44 º C, production of acid from glucose, xylose, galactose, manose, rhamnose and lactose (10, 11). In addition, APi20NE identification system was used to differentiate *Acinetobacter baumannii* from other species.

Screening for L-asparaginase production

All bacterial isolates were evaluated for their ability to produce L-asparaginase by culturing in M9 medium containing L-asparagine with phenol red as indicator and incubating at 37 º C for 24h. L-asparaginase producing colonies were selected on the basis of formation of pink zone around the colonies of the medium (9).

Determination of L-asparaginase activity

The active isolates were cultured on M9 broth supplemented with L-asparagine and phenol red as indicator, pH 7.0 and incubated at 37 ºC with shaking at 125 rpm for 24 h. L-asparaginase activity was measured following method of (12). The culture was centrifuged at 10000 xg for 15 min. This method utilizes the determination of ammonia liberated from L-asparagine in the enzyme reaction by the Nessler's reaction. Reaction was started by adding 0.5 ml supernatant into 0.5 ml of 0.04 M L-asparagine and 0.5ml of 0.05M tris-HCl buffer, pH 7.2 and incubated at 37ºC for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent (45.5 g HgI_2 and 35.0 g KI in 1 liter distilled water containing 112g of KOH) into tubes and incubated at room temperature for 15 min. After vortexing, the absorbance was measured at 500nm. A standard curve was drawn with various concentrations of ammonia.

Protein determination

Total protein was determined by using bovine serum albumin as protein standard and according to the method of (13).

Enzyme Purification

A liquots of cell-free dialysate (CFD) were separately treated with ammonium sulphate using range of saturation from 20 to 60 % , methanol , ethanol , acetone or isopropanol in a ratio of 1:1 , 2:1 , 3:1 , 4:1, or 5:1 (solvent :CFD). All samples were left overnight at 4ºC. The precipitates were collected by centrifugation at 9000 xg for 15 min, dissolved in 5 ml of 50 mMtris-HCl buffer, pH 7.5 and dialyzed overnight against the same buffer. The dialyzed fraction was applied to the column of CM Sephadex C-50 that was pre-equilibrated with 50mM tris-HCl buffer, pH 7.5. It was eluted with NaCl gradient (0.1-0.5M) and 0.1M borate buffer, pH 7.0. The active fractions (3ml/tube) were collected then L-asparaginase activity and protein concentration were measured.

Checking of enzyme homogeneity

The purity of the enzyme was checked by performing 12% SDS-PAGE as described by (14). Protein bands were visualized by staining with coomassie brilliant blue R-250. A reverse phase C-18 column (4.6×250 mm) of high performance liquid chromatography (HPLC) was also employed to test the enzyme purity. The sample components were separated using the solvent system acetonitrile- water (70:30) at flow rate of 0.5 ml/ min. A highly sensitive photo-diode array (PDA) detector (996 waters) was set to read the absorbance at 280 nm.

Characterization of the purified enzyme

1-Molecular weight determination

Molecular weight of the purified L-asparaginase was estimated by SDS- RAGE. SDS-PAGE was performed as described above using molecular markers kit .

2-Substrate specificity

To investigate the specificity of L-asparaginase, different substrate such as L-asparagine, D-asparagine, DL-asparagine, D-glutamine and L-glutamine were used. The substrates were prepared in tris- HCl buffer of pH 7.2(0.05) at 10 mM concentration. The reaction mixture was incubated for 30 min at 37ºC and asparaginase activity in each case was determined.

*3- Determination of kinetic parameter***s**

The michaelis constant (*Km*) and maximum velocity (*Vmax*) values of L-asparaginase were determined by measuring the rate of L-asparagine hydrolysis under standard assay conditions. The reaction mixture was 0.05M tris-HCl buffer, pH7.2 with L-asparagine substrate at concentrations ranging from 1.25 to 20 mg. The values of *Km* and *Vmax* were then determined from line weaver-Burk plot(15).

4-In vitro cytotoxicity assay of L-asparaginase

The *in vitro* cytotoxicity assay of the L-asparaginase was performed on human ovarian cancer cell line OAW-42.OAW-42 cell line was cultured in dulbeccos minimum essential medium (DMEM) with 10% FBS in a fully humidified air atmosphere containing 5% CO2 at 37 ºC. Cytotoxicity was measured using the MTT assay

, according to the method that described by (16) . Briefly, the cells (1×10^9) were seeded in each well containing 0.1 ml of medium in 96- well plates .After 24h , different test concentrations 3.0-9.0 mg/ml of L-asparaginase were added. The cells treated with L-asparaginase were incubated in CO_2 incubator with 95% humidity at 37 ºC for 24, 48 and 72 h. Cell viability was assessed by adding 10 µl/well of MTT (using 5 mg of MTT /ml of 0.05 M Tris-HCl, pH 7.2) . The plates were incubated at 37 ºC for additional 4 h. One hundred µl/well 0.04 M HCl in 2-propanol was directly added into the wells. After 30 min, the absorbance was measured at 570 nm by use of a ELISA microplate reader. Appropriate controls with no L-asparaginase but containing appropriate amount of tris-HCl buffer (used to prepare stock of L-asparaginase) and heat denatured enzyme were used as negative controls.

Results and Discussion

Isolation of bacteria

In an attempt to determine the distribution of *Acinetobacter* in nature , 44 samples of cucumber and lettuce plant rhizosphere soils were collected from an area approximately 150 miles long and 60 miles wide. Nine (20%) *Acinetobacter baumannii* isolates were obtained out of eleven *Acinetobacter* spp. Isolates. The nutritional properties of *Acinetobacter* and their ubiquitous occurrence in soil suggest that these organisms may be very important agents in the aerobic mineralization of organic matter in nature (17). The large majority of *Acinetobacter* strains normally occurring in water and soil do not require growth factors, and can grow in a mineralmedium containing acetate or some other organic sources of carbon and energy (18).

L-asparaginase production

Out of nine *Acinetobacter baumannii* isolates, eight isolates showed L-asparaginase activity on M9- asparagine agar plates with degradation of L-asparagine and releasing the ammonia then increasing in pH that detected by phenol red indicator which turns red from initial yellow color and formation of pink zone around the colonies. All active isolates were cultivated in M9 broth supplemented with L-asparagine. It was found that the range of L-asparaginase activity 0.31 to 2.72 U/ml (figure-1). *Acinetobacter baumannii* R7 was demonstrated high asparaginase activity of 2.72 U/ml. In the rhizosphere soil, root exudates are the natural source of amino acid for rhizoshere microorganisms, which may enhance L-asparaginase biosynthesis (19). In a study done by (20) revealed that medicinal plant rhizosphere soils can provide a rich source of L-asparaginase producing microorganisms. The presence of L-asparagine in the medium principle carbon source was induced of L-asparaginase producing bacteria (21). Kamble*et al.* (9) Showed that *E. coli, Aeromonas* spp., *Proteus* spp., *Serratia* spp., *Pseudomonas aeroginosa and Bacillus* spp. were isolated from soil and produced L-asparaginase.

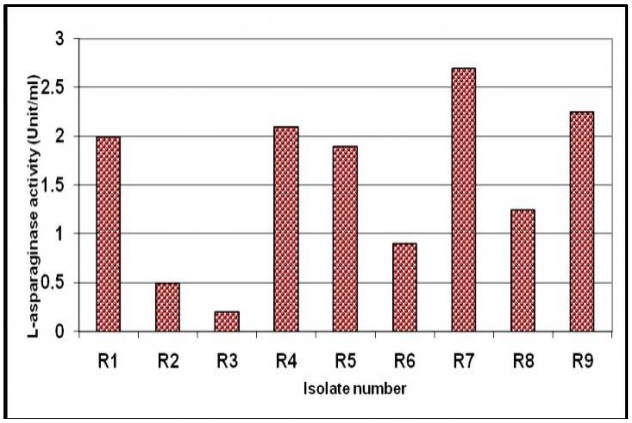

Figure (1) L-asparaginase production by *Acinetobacter baumannii* isolates.

Purification of L-asparaginase

The first step of purification of L-asparaginase is carried out by precipitation of protein from the cell-free dialysate. Isopropanol (1:2) was found to be superior in obtaining protein fraction having the highest specific activity .This resulted in 18.3 fold of L-asparaginase purification with a yield of 86.2% (table-1). A summary of purification steps of L-asparaginase was recorded in (table-2). The precipitated enzyme was then purified by Ion exchange through CM-Sephadex C-50.In this step, L-asparaginase was purified 92.9 fold with a yield of about 77% and specific activity of 2.78 U/mg protein. The activity was located in peak 2 (Figure-2).

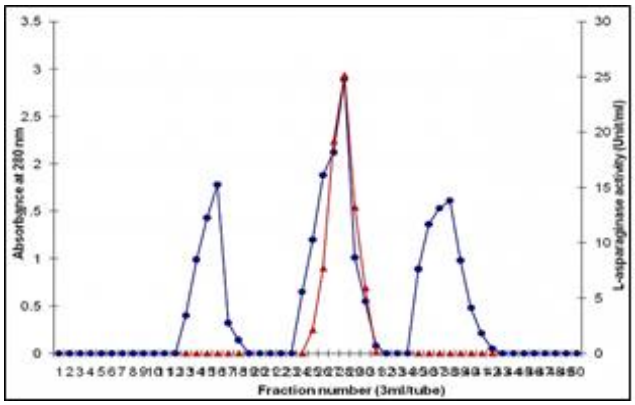

Figure (2): Purification of L-asparaginase produced from *Acinetobacter baumannii* R7 using ion exchange on CM Sephadex C-50. (●) refer to protein, (▲) refer to L-asparaginase activity

The purity of the purified enzyme was checked by SDS-PAGE and reverse phase HPLC on C-18 column. The purified enzyme showed a single band in SDS-PAGE gel indicating that it was homogenous (Figure-3).

HPLC chromatogram of the purified enzyme also revealed a single peak at a retention time of 2.5 min confirming that it was a pure preparation (Figure-4). Devi and Azmi (16)

Table (1): Precipitation of L-asparaginase from *Acinetobacterbaumannii*R7 cell-free dialysate by ammonium sulfate and low molecular weight alcohols

Fold of purification	Yield %	Specific activity (U/mg)	Total protein (mg/ml)	Total activity (U/ml)	Treatment	
1	100	0.03	97.18	2.72	**Cell-free filtrate**	
0.68	93.6	0.02	97.02	2.68	**Cell-free dialysis**	
6.66	20	0.2	23	4.71	**20**	**$(NH_4)_2So_4$(%)**
9	41.1	0.27	25	6.82	**30**	
10	68.6	0.3	37	11.21	**40**	
13	93.5	0.39	39	15.27	**50**	
4.6	37.5	0.14	42	6.12	**60**	
7.3	20.2	0.22	14.4	3.31	**1:01**	**Methanol (vol/vol)**
7.6	36.2	0.23	25.1	5.91	**2:01**	
15.6	85	0.47	29	13.88	**3:01**	
9.6	58.2	0.29	31.8	9.5	**4:01**	
4	25.7	0.12	34.6	4.21	**5:01**	
0.1	27.6	0.004	11.11	4.51	**1:01**	**Ethanol (vol/vol)**
9.3	36.7	0.28	21	5.99	**2:01**	
11	51.58	0.33	25.3	8.41	**3:01**	
14.3	8.4	0.43	31.66	13.71	**4:01**	
17	61.9	0.51	33.05	10.11	**5:01**	
6	19.4	0.18	17.21	3.18	**1:01**	**Acetone (vol/vol)**
7.3	25.8	0.22	17.06	4.22	**2:01**	
11.6	51.3	0.35	23.3	8.38	**3:01**	
13	77	0.39	31.55	12.57	**4:01**	
5.6	37.5	0.17	34.7	6.12	**5:01**	
16.3	44.2	0.49	14.5	7.22	**1:01**	**Iso-propanol (vol/vol)**
19	83.1	0.52	26.13	13.66	**2:01**	
18.3	86.2	0.48	29.01	14.07	**3:01**	
14	80.7	0.42	31.01	13.18	**4:01**	
10.6	73.5	0.32	36.5	12	**5:01**	

Table (2): A summary of treatments used for the purification of L-asparaginase from *Acinetobacterbaumannii* R7

Yield (%)	Fold of purification	Total activity	Specific activity (U/mg)	L-asparaginase activity (U/ml)	Total Protein (mg/ml)	Size (ml)	Step
100	1	163.2	0.03	2.72	97.18	60	**C.F.F.***
93.6	0.6	152.7	0.02	2.68	97.02	57	**C.F.D. ****
86.2	18.3	136.6	0.52	13.66	26.13	10	**C.F.P.** **
77	92.9	125.7	2.78	25.15	9.02	7	**CM- Sephadex C-50**

*Cell – free filtrate **Cell – free dialysate ***Cell – free precipitate

reported that 4.7 fold purification and 24% a yield of L-asparaginase from *Erwinia carotovora* by sulphopropyl sephadex chromatography. The partially purified L-asparaginase from *E. coli* had the purification fold of 2.15 with 20% yield after 20-40 % ammonium sulphate precipitation (22). The purification of L-asparaginase from *Pseudomonas aeroginosa* by sephadex G-100 gel filtration and SDS-PAGE analysis of the protein was performed by (23). Ameni*a et al.* (24) purified the L-asparaginase from *Streptomyces gulbargensis* with 82.12 purification fold and yield 32% after simultaneous purification steps of ammonium sulphate precipitation, sephacryl S-200 gel filtration and CM sephadex C-50 chromatography.

Characterization of the purified enzyme

1-Determination of the molecular weight

SDS-PAGE of the purified enzyme was revealed only a single distinctive protein band of L-asparaginase with an apparent molecular weight of 160 kDa (Figure-3). The molecular weight of *E. coli* L-asparaginase determined by ion exchange chromatography was 153 kDa (25).L-asparaginase from *Pseudomonas aeroginosa* 50071 showed single protein band with a molecular weight of 160 kDa (23). The partially purified enzyme from *Bacillus circulans* showed an a proximal molecular weight of 140 kDa (26) , but the enzyme of *Erwinia carotovora* exists in the form of dimmer of dimmers with molecular weights of 40.2 and 39.8 kDa (16).

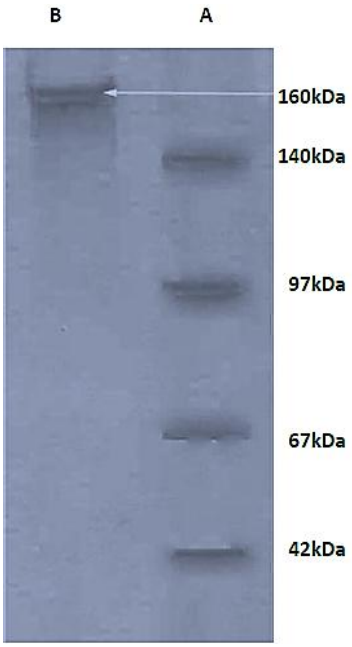

Figure (3): PAGE-SDS of L-asparaginase from *Acinetobacter baumannii* R7. Electrophoresis was carried out on a 12% polyacrylamide and stained with Coomassie blue R-250. Lane A included the molecular weight of the marker proteins. Lane B contained CM- SephadexC50 column purified enzyme.

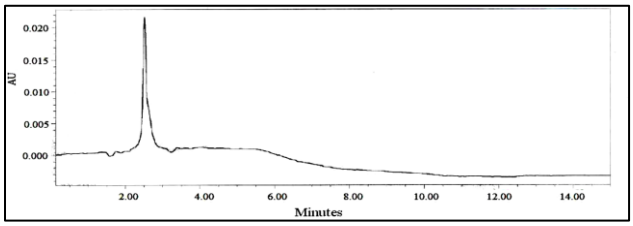

Figure (4): HPLC profile of the purified L-asparaginase from *Acinetobacterbaumannii* R7 using a reverse phase C-18 column (4.6 x 250mm). The purified enzyme (10 µl) was injected into the loop of column and eluted with acetonitrile-water (70:30) at a flow rate of 0.5 ml/min. Aphoto-diode array (PDA) detector was set to read the absorbance at 280nm.

Characterization of the purified enzyme

1-Determination of the molecular weight

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2-Substrate specificity

The substrate specificity of the enzyme is presented in (Figure-5). The results revealed that the highest apparent affinity of L-asparagine was found towards its natural substrate L-asparagine while no activity could be detected against L- glutamine and D- glutamine .This property of the enzyme is very essential on the treatment of patients were incomplete removal of asparagine is required . The observations reported under the present study are in good agreement with the finding of (27).

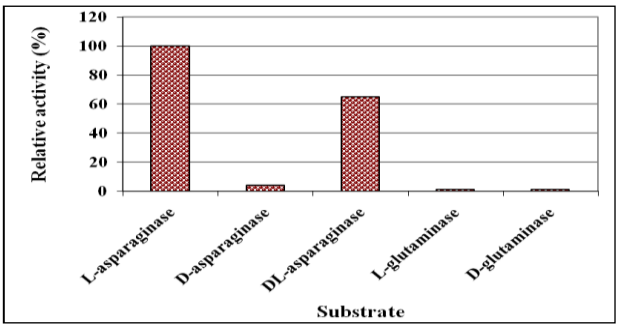

Figure (5): Substrate specificity of purified L-asparaginase from *Acinetobacter baumannii* R7

3-Determination of kinetic parameters

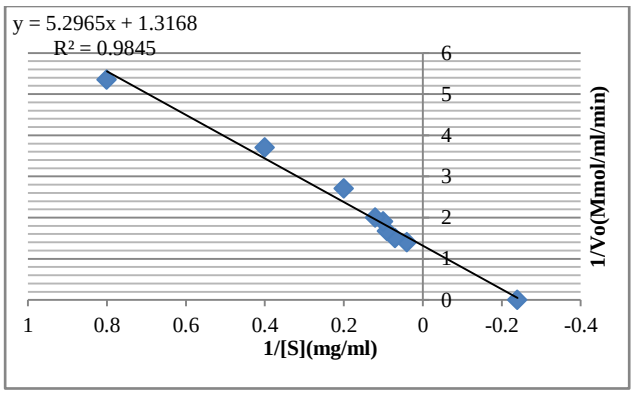

Figure (6): Lineweaver-Burk plot of L- asparaginase fraction from *Acinetobacterbaumannii* R7.The L-asparaginase fraction was incubated with different concentrations of L- asparagine (1.25 to 20 mg) as substrate.

The Linewearer- Burk plot of the L-asparaginase activity at 30 min and 37ºC in 0.05 M tris-HCl buffer, pH 7.2 with various concentrations of L-asparagine as the substrate is shown in (Figure-6). The relationship was linear(R2=0.984), giving estimate of the $K_m$and V_{max} values of the enzyme as 0.421 mg/ml and 0.826 U/mg of protein, respectively, and this indicates the high affinity of the enzyme to the substrate. L-asparaginase of different microorganisms has different substrate affinities and probably plays different physiological roles in the enzyme activity. Lower K_m value (0.074mM) was obtained for L-asparaginase from *Vibrio costicola* (28). Also K_m and V_{max} values of L-asparaginase from *Pseudomonas*

*aeruginosa*50071 were 0.147 mM and 35.7 IU, respectively(23).

4-In vitro cytotoxicity assay of L-asparaginase

The purified L-asparaginase enzyme from *Acinetobacter baumannii* has been subjected to cytotoxic activity *in vitro* on the OAW-42 cell line for different times . The treatment of cancerous cell line with increasing concentration of L-asparaginase (up to 9.0 mg/ml) resulted in appreciable inhibition of cell growth (59 and 51% survival) for 24 and 48 h ,respectively, when compared to controls (Figure -7) .This study indicates that this enzyme has potential for being used as anticancer drug for treatment of human ovarian cancer. Apoptosis of leukemia cells induced by L-asparaginase was found to be an event that has been associated with the cell cycle arrest in G1phase (29). Cappelleti*et al*. (30) studied *in vitro* cytotoxicity of a novel L-asparaginase from the pathogenic strain *Helicobacter pylori* CCUGI 7874 against different cell lines and reported that AGS and MKN28 gastric epithelial cells being the most affected.

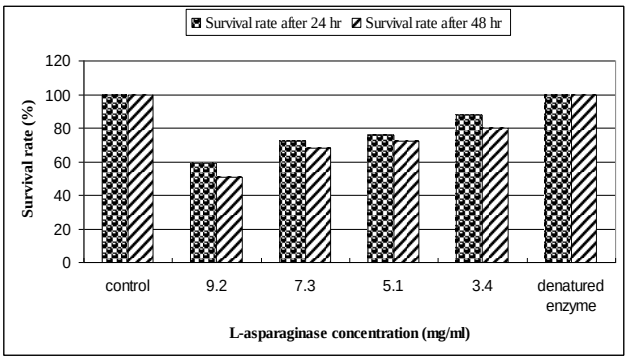

Figure (7): Cytotoxicity of the purified L-asparaginase from *Acinetobacter baumannii* R7

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