Purification, Characterization and Antitumor Activity of L-asparaginase from Chicken liver

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Abstract: Abstract: The L-asparaginase (E.C.3.5.1.1) produced by chicken liver was isolated and characterized. Different purification steps (including ammonium sulphate fractionation followed by separation on Sephadex G-100 gel filtration and Sephadex G-200 gel filtration) were applied to crude filtrate to obtain a pure enzyme preparation. The enzyme was purified 128.5 ± 0.5 fold and showed a final specific activity of 158.11 ± 5.0 U/mg with a 17.1 ± 8.6 % yield. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme revealed it was one peptide chain with M_r of 33 kDa while by gel filtration appears to be 36 kDa. The enzyme was very specific for L-asparagine and doesn't hydrolyze L-glutamine. A Lineweaver-Burk analysis showed a K_m value of 1.66 mM toward L-asparagine as substrate and V_{max} of 34.47 U. The enzyme showed maximum activity at pH 9.5 when incubated at 60°C for 20 min. The amino acids composition of the purified enzyme was also determined. Antitumor activity was investigated. The enzyme inhibited the growth of the two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (Hct-116) with IC_{50} value of 8.38µg/ml and 4.67µg/ml, respectively. While IC_{50} was greater than 10µg/ well for MCF7 (breast carcinoma) cell line.
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1. Introduction:

L-asparaginase amidohydrolase (E.C. 3.5.1.1) is an enzyme which catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia according to the following equation: L-asparagine +H_2O------->L-aspartate+ ammonia.

It is widely distributed in nature, not only in animal organs such as liver of guinea pig, placenta, kidney and intestine of beef and horse (Prista and Kyridio, 2001), but also in microorganisms such as *Escherichia coli*, *Thermus thermophius* , *Erwinia caratovora* (Kotizia and Labrou, 2005; Michalska, *et al.*, 2006; Kotzia and Labrou, 2007; Verma *et al*., 2007 and Tabandeh and Aminlari, 2009) and also in plants such as soy beans, *Oryza sativa, Hordenum vulgare* and *Lupinus* species (Borek and Jaskolski, 2001).

Interest in this enzyme arose a few decades ago when it was discovered that the antilymphoma activity of whole guinea pig serum was result of the enzyme L-asparaginase (Prista and Kyridio, 2001). The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine. As in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine (Narta *et al*., 2007). The discovery of new L-asparaginase serologically different but having a similar therapeutic effect is highly desired, (Moharam *et al*., 2010). The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma (Tabandeh and Aminlari, 2009 and Sunitha *et al*., 2010).

Little work has been carried out on L-asparaginase from chicken liver. The present paper is devoted to the purification of an asparaginase from chicken liver and to a comparative study of some of its biochemical and biological properties.

2. Materials and methods

Chemicals:

Anhydrous L-asparagine , trichloroacetic acid, Nessler reagent chemicals (HgI_2, KI and sodium hydroxide, molecular weight markers for gel filtration, all resins and reagents for electrophoresis were obtained from Sigma chemical CO. (St Louis, Mo). Sephadex G-100, Sephadex G-200 for chromatography and molecular weight markers for SDS-polyacrylamide gel electrophoresis were obtained from Pharmacia Fine Chemicals (Sweden). Buffers were prepared according to the method of Gomori (1955), and the final pH values were checked on Hanna pH meter. All the other chemicals were analytical grade.

Animals:

The screening was carried out with different animal's serum and organs such as liver, lung, kidney, testis, ovaries, heart, pancreas, spleen and brain from mouse, rabbits, chicken, buffalos and rats. Where the chickens and rabbits were brought from markets. Buffalos were brought from EL-Bassatein's slaughter house and finally rats and mice were brought from animal's house in National Research Center, Giza.

The enzyme activities of homogenates of organs and supernatants of serum from different species (crude enzyme) with different buffers and molarities that are commercially available in a large quantity were studied in comparison with liver homogenates of laboratory animals.

All experiments were carried out with chicken livers. They were obtained from animals of random breed and sex, maintained from markets, liver was kept frozen at - 40°C.

L-asparaginase assay:

The enzyme activity was assayed according to Wriston (1970) method. The reaction mixture contained 0.9 ml of 0.01mole L-asparagine preparation in 0.05 mole sodium borate buffer, pH 8.5 and adequate amount of L-asparaginase was incubated for 20 min at 37°C. The reaction mixture was centrifuged at 6000 xg for 10 min and the ammonia released in the supernatant was determined by Nesslerization reaction. In brief, to 0.5 ml of supernatant, 1.75 ml distilled water, 0.25 ml of Nessler reagent was added. After 10 min; absorbance at 480 nm were read with appropriate control.

One enzyme unit (U) is defined as the amount of enzyme that librates one µmole of ammonia per min at 37°C. Standard curve of ammonium sulphate was used for calculating ammonia concentration. The activity values of samples present in the paper were average values of three repeated measurements. Where the specific activity is defined as the units of L-asparaginase per milligram protein (Bansal *et al.*, 2010).

Protein determination:

The total protein contents of the samples were determined according to the method described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Purification of enzyme:

The purification was carried out at 4°C on the crude enzyme by:

1. Ammonium sulphate precipitation:

Certain volume of the prepared crude enzyme was treated with different concentration of ammonium sulphate (20-60%). The mixture was left at 4°C over night, follwed by centrifugation at 13000 r.p.m for 15 mins at 4°C. The resulting precipitates were dissolved in appropriate amount of distilled water and dialyzed exhaustively against distilled water for 2 days at 4°C to get ride of the excess of ammonium sulphate.

2-Sephadex G-100 gel filtration:

The dialyzed ammonium sulphate fraction was applied to Sephadex G-100 column (1.2 x55 cm) was pre-equilibrated with 0.01 M sodium borate buffer pH 8.5 at a flow rate of 20 ml/h. The fraction were collected and examined for enzyme activity and protein content.

3-Sephadex G-200 gel filtration:

The fraction from Sephadex G-100 with high L-asparaginase activity was loaded onto the pre-equilibrated Sephadex G-200 column (1.2 x 55 cm) with 0.01 M sodium borate buffer, pH 8.5 at a flow rate of 16 ml/h. The fractions were collected and examined for enzyme activity and protein content.

Native-PAGE: a slab gel electrophoresis was carried out using a 15% poly-acrylamide gel (pH 6.2). After electrophoresis in a tris-glycine buffer (pH 8.3) at 200V for 7h at 70°C, the proteins in the gel were stained with coomassie brilliant blue R-250 and destined (EL-Gamal et al., 2001).

Molecular weight determination by:

1- SDS-PAGE: was performed following the method of (Laemmli, 1970) with separating acrylamide gel 12.5% (wt/vol) and stacking gel 3% (wt/vol) containing 0.1% (wt/vol) SDS. The log molecular weight of different standard molecular weight marker proteins of 66 kDa (bovine serum albumin), 45 kDa (egg albumin), 36 kDa (glyceraldehyde-3-p-dehydrogenase), 29 kDa (carbonic dehydrogenase bovine), 24 kDa (trypsinogn bovine pancrease), 20 kDa (trypsin inhibitor soybean) and 14.2 kDa (∞-lactoalbumin bovine milk) were plotted against their relative mobility in the gel, and from the plot the molecular weight of the protein was calculated. The gel was stained with Coomassie brilliant blue R-250.

2-Gel filtration: the molecular weight of the purified enzyme was estimated by gel filtration chromatography through a column (1.2 x 40 cm) of Sephadex G-200 as described by Andrew (1964), pre-equilibrated with 0.01 M sodium borate buffer pH 8.5. The column was calibrated with standard molecular weight marker proteins as: 66 kDa (bovine serum albumin), 33 kDa (trypsin from porcine pancrease), 29 kDa (carbonic anhydrase), 20.1 kDa (trypsin inhibitor), and 14.2 kDa (lyzozyme).

Amino acid composition:

Purified L- asparaginase was dissolved in one ml of dilution buffer/Eppendorf-Germany, and then

injected into full automated amino acid analyzer, eppendorf LC 3000**.**

The conditions were estimated to be flow rate 0.2 ml/min**,** pressure of buffer form 0 to 50 bar, pressure of reagent to 0-150 bar and reaction temperature (123°C).

Antitumor activity:

Potential cytotoxic activity against some tumor cell line was performed in the National Cancer Institute using method of Skehan *et al.* (1990).

Prepared L-asparaginase partially pure (ammonium sulphate) and pure enzymes were lyophilized. One milligram of each lypholized powders were dissolved in 0.1 ml of DMSO and the volume completed to one ml with distilled water.

Cells were plated in (10^4 cells/well) for 24 h before treatment with the dried L-asparaginase to allow attachment of cell to the plate.

Different concentrations of the compound under test (0, 1, 2.5,5,10 µg/ml) were tested. Triplicate tested were prepared for each individual dose.

Monolayer cells were incubated for 48 h at 37°C in atmosphere of 5% CO_2, after 48 h cells were fixed, washed stained with sulforhodamine-B stain. Excess stain was washed with acetic acid and attached stain was recovered with tris EDTA buffer. Color intensity was measured in an ELISA reader.

The relation between surviving fraction and the drug concentration is plotted to get the survival curve of each tumor cell line for the specified enzyme.

The effective dose required to inhibit cell growth by 50% (IC_{50} µg/ml) was determined. Doxorubicin was used as positive control.

Dried L-asparaginase was tested for the following tumor cell lines at concentration between 1.0-10.0 µg/ml:

Hepatocellular carcinoma cell line.

Breast carcinoma cell line.

Colon carcinoma cell line.

3. Results and Discussion.

L-asparaginase of the liver of various species:

The enzyme activities of homogenates of liver from different species that are commercially available in large quantities were studied in comparison with liver homogenates of laboratory animals and liver of buffalo. The results are shown in table (1). The result encouraged the use of chicken liver for the further studies as it has a higher L-asparaginase activity, 8.66 U/gm of liver than the other livers with specific activity, 0.648 U /mg at optimum assay condition.

Preparations of the enzyme from chicken liver:

Liver was homogenized with sand and extracted in twice their volume of 0.01 M sodium phosphate buffer, pH 7.4 containing 0.1 M potassium chloride. They were incubated separately with L-asparagine dissolved in 0.05 M sodium borate buffer, pH 8.5 at 37°C for 30 mins.

Purification of L-asparaginase enzyme from chicken liver:

Many steps commonly employed for enzyme purification were inapplicable. The enzyme activity (L-asparaginase) was destroyed by organic solvents (acetone precipitation). DEAE-cellulose column could not be employed successfully owing to the low stability of the enzyme at salt concentrations.

The partial purification of the L-asparaginase crude extract that was affected by the ammonium sulphate (20-60%) saturation showed that most of the enzyme activity was preserved in the precipitate. The total protein concentration was decreased from 238 ± 1.9 to 64 ± 0.46 mg in ammonium sulphate precipitate with 55.3±1.2 % yield.

Fig. (1) shows the elution profile of purification of the ammonium sulphate fraction (20-60 %) on Sephadex G-100 column. This fraction contained wide peak with L-asparaginase activity with specific activity 18.2 ± 2.2 U/mg.

The elution profile of the most active fraction, collected from Sephadex G-100 on Sephadex G-200 column was illustrated in fig (2).

Although this fraction contained three different protein peaks, only one peak showed L-asparaginase. A sharp distinctive peak of L-asparaginase activity which fits with only one protein peak was obtained (tubes number 12 and 13) as shown in fig.(2). The various steps of the purification procedure finally adopted by a relative simple method and are shown with summarizing data in table (2). The activity values were average values of nine repeated purification batches.

Thus, purification of L-asparaginase to homogeneity from chicken liver was achieved by simple steps with final yield 17.1 ± 8.6 %, a purification fold 128.5 ± 0.5 and a specific activity of 158.11 ± 5.0 U/mg protein.

Table (1): L-asparaginase activity in the livers of various species:

Species	Wet weight/Volume (gm/ml)	L-asparaginase Activity (U/ml)	Total units	Total L-asparainase activities (U/gm) of liver	Protein conc. (mg/ml)	Specific activity (U/mg)
Mouse	2.19/7	0.0	0.0	0.0	7.26	0.0
Rabbit	5.04/8.2	1.478	12.12	2.40	5.240	0.28
Chicken	4.42/6	6.384	38.30	8.66	9.85	0.648
Buffalo	9.50/18.9	2.497	47.20	4.96	11.10	0.224
Rat (female)	11/22	2.077	45.69	6.06	-	-
Rat (male)	7.39/20.1	1.182	23.76	3.21	7.990	0.147

Note: 0.05 M sodium borate buffer pH 8.5 at 37°C for 10 min.

Table (2): Purification profile of L-asparaginase from fresh chicken liver (10 g).

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	284±2.4	238 ±1.9	1.23±0.1	1.0	100
Ammonium sulphate fraction (20-60%)	158±1.0	64±0.46	2.47± 0.3	2.0 ± 0.3	55.3 ±1.2
Gel filtration on Sephadex G-100	100 ±7.0	5.53 ±0.09	18.2 ±2.2	14.8 ±0.23	34.8 ±2.0
Gel filtration on Sephadex G-200	48 ± 3.0	0.305 ±0.02	158.11 ±5.0	128.5 ±0.5	17.1± 8.6

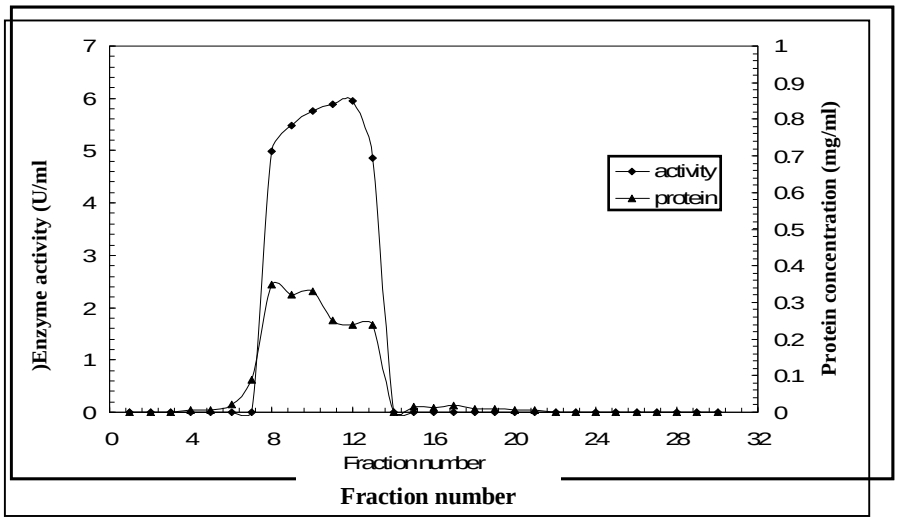

Fig. (1): Elution profile of L-asparaginase on Sephadex G-100 column.

The dialyzed ammonium sulphate precipitate (20-60%) was chromatographed on Sephadex G-100 in (1.3x 55 cm) column. The column was equilibrated and eluted with 0.01 M borate buffer pH 8.5. The fractions were assayed for L-asparaginase activity and protein content.

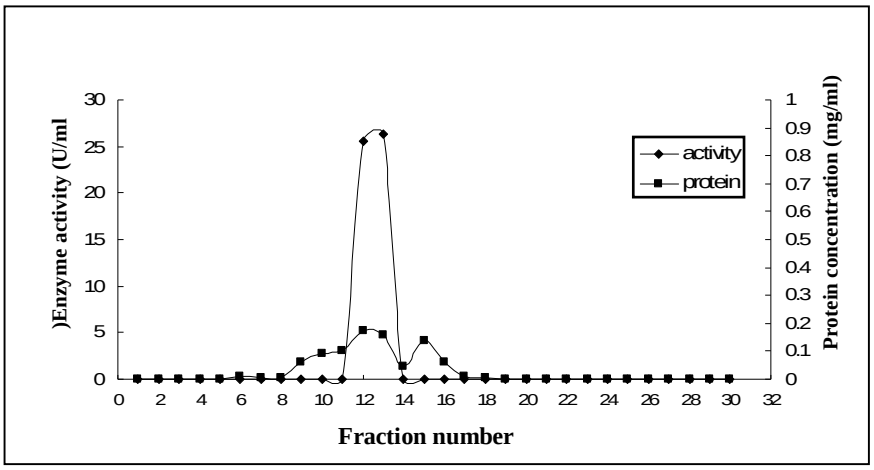

Fig. (2): Elution profile of L-asparaginase on Sephadex G-200 column.

The most active collected fraction from Sephadex G-100 was applied to Sephadex G-200 (1.2 x 55 cm). The fractions were assayed for the enzyme activity and protein content.

Molecular weight of L-asparaginase (Figure 3):

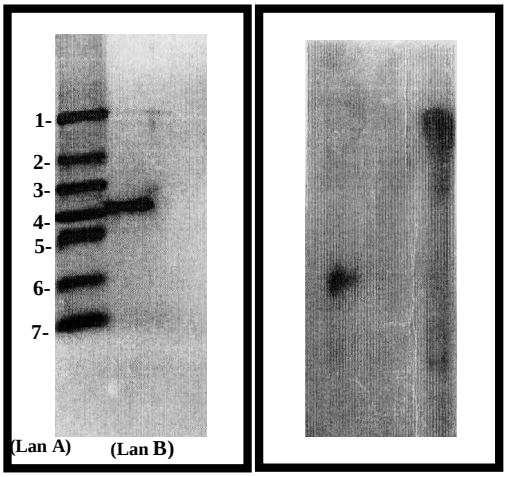

Fig. (3a&b): Native and PAGE –SDS of L-asparaginase from chicken liver.

Lane A: Included the following standard proteins:

1- Bovine serum albumin (66,000).
2- Egg albumin (45,000),
3- Glyceraldehyde-3-p-dehydrogenase (36,000),
4- Carbonic dehydrogenase bovine (29,000),
5- Trypsinogn bovine pancrease (24,000),
6- Trypsin inhibitor soybean (20,000) and ∞-lactoalbumin bovine milk (14,200)
7- Lane B: Purified enzyme (5µg).

Native-PAGE of the purified enzyme preparation from Sephadex G-200 column was performed to get basic information about the purity of the L-asparaginase. It was revealed only one distinctive band as shown in fig. (3a).

SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), was performed with the purified enzyme. The result revealed to no detectable contamination and a single distinct band was observed with molecular weight of about 33 kDa, fig (3b).

Determination of molecular weight of L-asparaginase by gel filtration (Sephadex G-200): The molecular weight of the purified enzyme is found to be 36 kDa. By using different standard proteins with known molecular weights, it was found that the apparent molecular weight of chicken liver L-asparaginase preparation was 36 kDa, fig. (4).

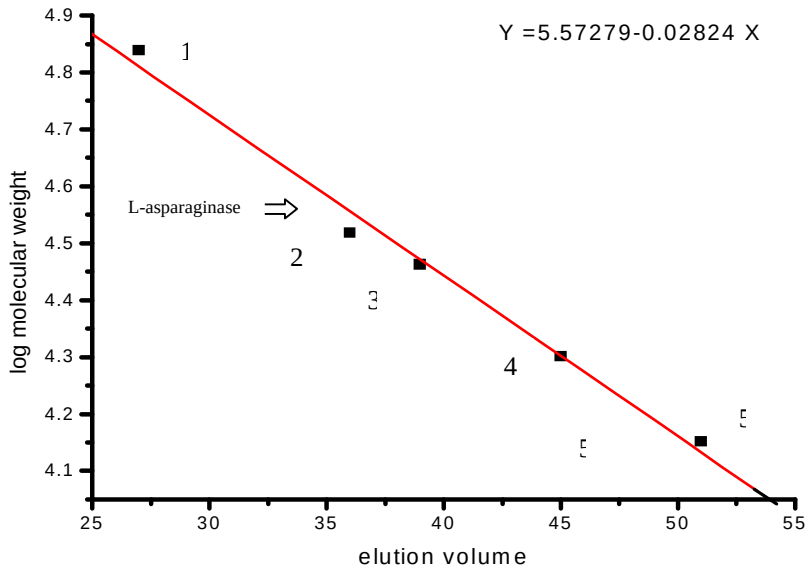

Fig. (4): Determination of the molecular weight of the purified L-asparaginase by gel filtration on Sephadex G-200 column (1.2 X 40 cm). 1) Bovine serum albumin (66,000) 2) trypsin from porcine (33,000) 3) carbonic anhydrase from bovine erythrocytes (29,000), 4) trypsin from soybean inhibitor(20,000), 5) lysozyme (14,200).

In this respect, the enzyme was approximately similar to that obtained from *Pseudomonas stutzeir* MB-405, *Thermus thermophilus* and *Eshirichia coli* with M_r range from 33-34 kDa, (Manna *et al.*, 1995; Prista &Kyridio, 2001 and Soares *et al*., 2002). While the enzyme was lower than that obtained from *Pisum sativum* (Sieciechowicz *et al.*, 1989). M_r of L-asparaginases isolated from *Pseudomonas aeruginosa* 50071 and *Chlamydomonas* sp were approximately 160 kDa, (EL-Bessoumy *et al*., 2004 and Dhevagi and Poorani, 2006).

Physicochemical properties of the purified L-asparaginase:

The pH influence on the L-asparaginase activity was studied using a 0.05 M borate buffer of different pH values ranging from 4 to 11.5. The enzyme activity gradually increased until pH 7.5 and remains high active over a wide range of pHs' from 7.5 to11 and at which the maximum activity was observed, fig (5). At higher pH than pH 11, the enzyme activity was decreased to 33.3%. A similar pHs' values were obtained from Guinea pig serum, Pseudomonas stutzeir MB-405 and from Helicobacter pylori (Tower et al., 1963; Manna et al., 1995 and Cappelletti et al., 2008).

The effect of the incubation time on L-asparaginase activity was studied in the ranges of 5 to 180 min, (Fig.6). L-asparaginase activities increased as the incubation time increased. The activity ran at maximum for 30 minutes and still maximum for 90 min. After 90 min, it decreased as the time increased.

The reaction rate of L-asparaginase was measured at various temperatures from 40 to 75°C, (Fig.7). It appears that L-asparaginase optimally deamidated at 60°C. At higher temperature than 60°C the reaction rate

declined to 77.2% of activity 75°C. When the enzyme was exposed in absence of the substrate to 30°C up to 45°C for 60 min, then their activities were measured, as described before the activity was about 52 % increased, (Fig.8). Beyond this temperature the enzyme becomes increasingly unstable. Similar results were recorded for asparaginase from *Pencillium politans NRC 510* (Tower *et al*., 1963 and Ali and EL-Sayed, 2006). They were proved that *Thermus thermophilus* and Guinea pig serum L-asparaginas, are quite stable and linear even at 70°C or 77°C. On the other hand, L-asparaginase from *Erwinia* sp had a maximum activity at 35°C (Borkotaky and Bezbaruah, 2002).

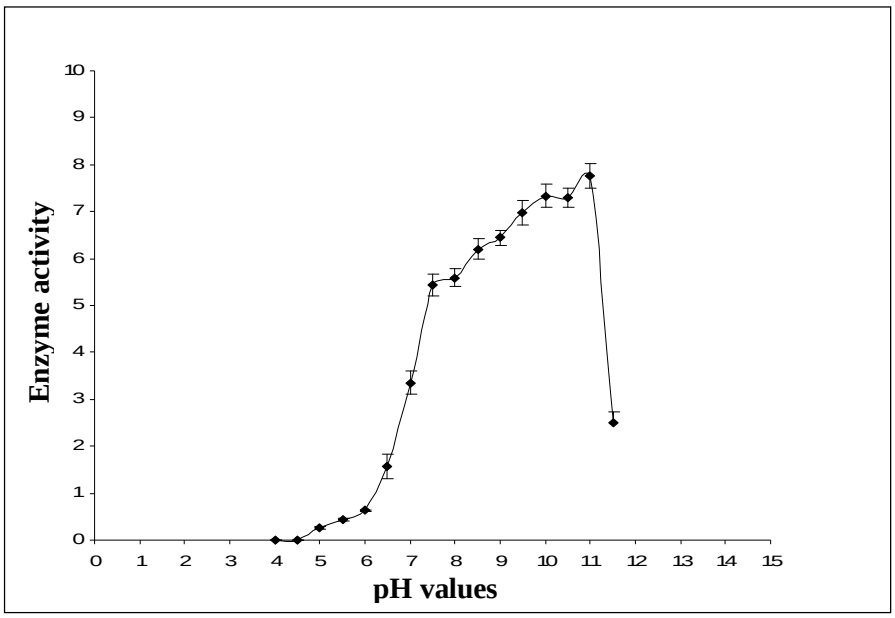

Fig. (5): Effect of pH on L-asparaginase activity.

The purified L-asparaginase was very specific for L-asparagine and low for DL-asparagine (22.5%) and did not hydrolyzed L-glutamine. L-asparaginase of different microorganism has different substrate affinities and probably plays a different physiological role in the enzyme activity, (EL-Bessoumy *et al*., 2004).

The K_m value of the purified enzyme was determined according to the method of Lineweaver and Burk (1934). A Lineweaver-Burk analysis gave K_m of 1.66 mM toward L-asparagine as substrate and the maximum velocity (V_{max}) of 34.47 U (Fig.9). Higher K_m values (6.6 and 7.0 mM) for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively has been reported by Chang and Franden (1981). On The other hand, a lower K_m value (0.058 mM) was obtained for L-asparaginase from *Erwinia chrysanthemi* 3937 *(*Kotzia and Labrou, 2007).

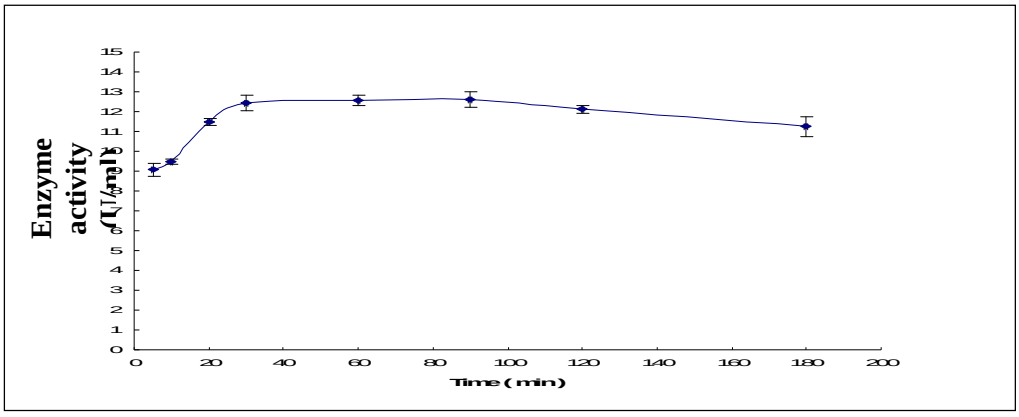

Fig. (6): Effect of time on the pure L-asparaginase activity.

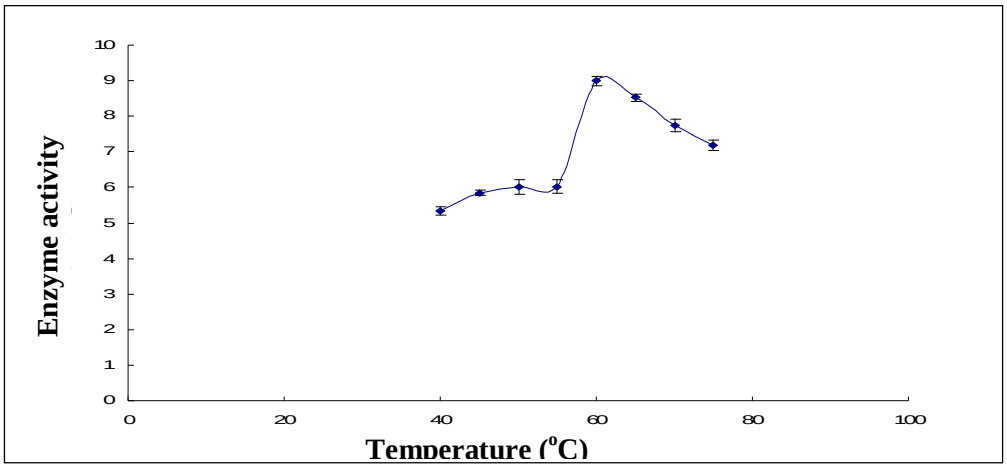

Fig. (7): Effect of temperature on L-asparaginase activity.

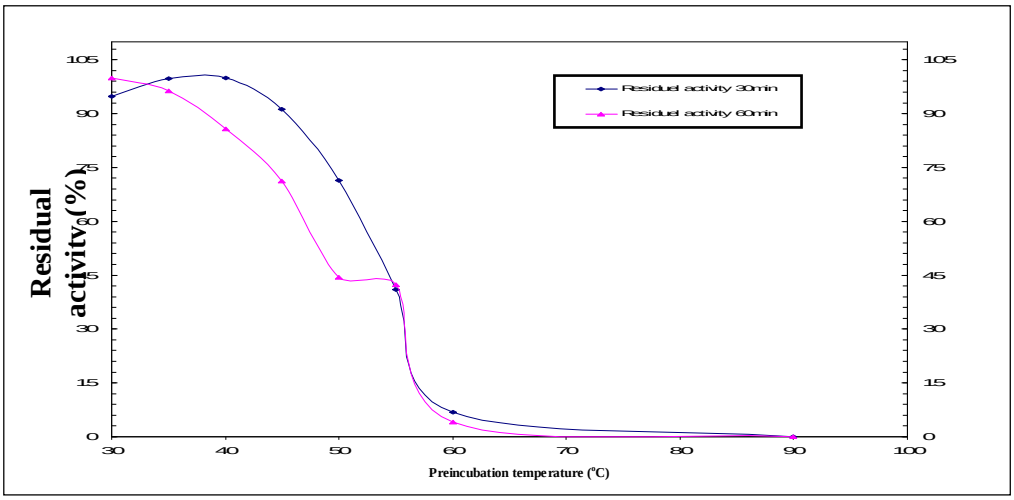

Fig. (8): Thermal stability of L-asparaginase activity.

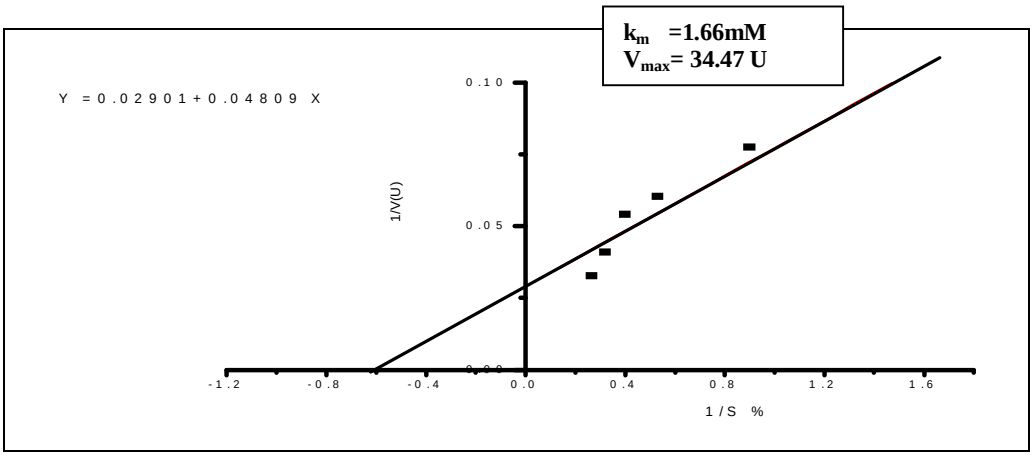

Fig. (9): Lineweaver –Burk plot of L-asparaginase activity using L-asparaginase as substrate.

Amino acid composition:

Table (3) shows the amino acid contents of the purified chicken liver L-asparaginase.

Table (3): Amino acid contents of the purified chicken liver L-asparaginase.

Amino acid concentration (mol%)	Amino acid
12.64	Aspartic acid
2.03	Threonine
2.35	Sereine
6.48	Glutamic acid
2.30	Glycine
4.65	Alanine
7.73	Cystin
0.095	Methionine
2.43	Isoleucine
4.64	Leucine
1.22	Tyrosine
1.71	Phenylalanine
1.39	Histidine
2.78	Lysine
1.86	Arginine
0.39	Proline

The quality of chicken liver L-asparaginase was assessed for its amino acid contents. The purified enzyme was rich in aspartic acid, glutamic acid and cystin. **Qian *et al*. (1996)** reported that aspartic acid protects the active site of *Esherichia coli* L-asparaginase.

Biological properties:

The lypholized L-asparaginase enzyme (partial and pure) was subjected to cytotoxic activity *in vitro* on the cell lines available HEPG2 (hepatic carcinoma), HCT (colon carcinoma) and MCF7 (breast carcinoma) using SRB assay. The growth inhibition data were expressed as percent of control. Results in figs. (10 and 11) shows that no significant differences were observed in the cytotoxicity between the highly purified and partially purified L-asparaginase enzyme against HEPG2 (hepatic carcinoma) cell line (IC_{50} = 8.38 µg /well and 8.91 µg / well respectively). While results showed difference between the cytotoxicity of highly purified and partially purified L-asparaginase enzyme against HCT (colon carcinoma) cell line (IC_{50} = 4.67 µg / Well and 6.44 µg /well respectively).While IC_{50} was greater than 10µg/ well for MCF7 (breast carcinoma) cell line. The sensitivity of MCF, HEPG2 and HCT cells to both asparaginases (partial and pure fraction) appeared to be dose dependent, resulting in significant decrease in viable cells. Treatment of different tumor cancer cell lines with increasing the concentrations of L-asparaginase up to10 µg results in appreciable inhibition of the cell growth.

Cappelletti *et al*. (2008) studied *in vitro* cytotoxicity of a novel L-asparaginase from the pathogenic strain *Helicobacter pylori* CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected. While in breast cell line used in this investigation do not contain L-asparagine synthetase activity (Prista *et al*., 2001).Therefore, the selective growth inhibition by L-asparaginase of breast cancer cell could be related to the absence of intracellular L-asparagine synthetase activity in this cell.

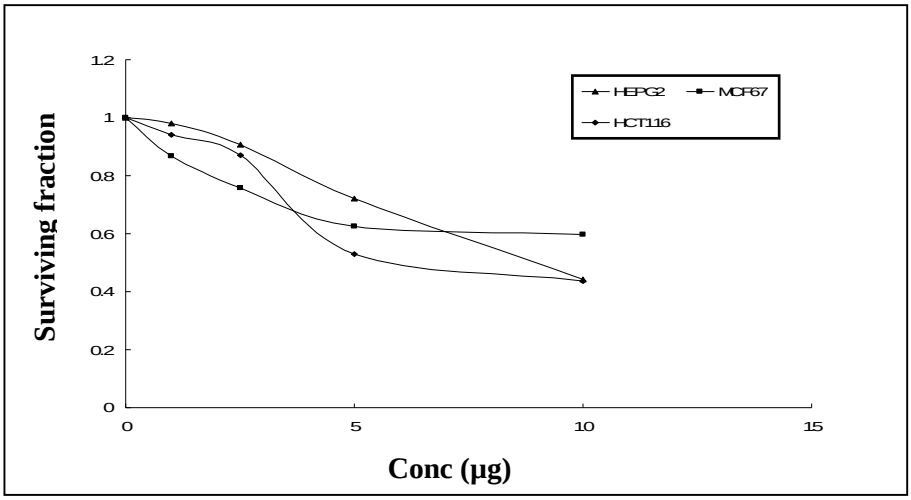

Fig (10): Cytotoxic activity of partially pure L-asparaginase.

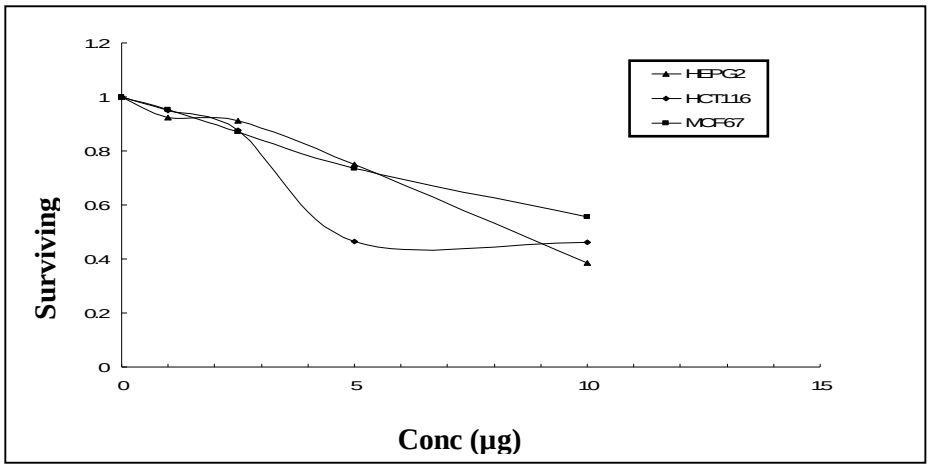

Fig (11): Cytotoxic activity of pure L-asparaginase.

Our results showed that the purified L-asparaginase from chicken liver has got the favorable activity at wide range pH, high temperature, high affinity towards L-asparagine, no glutaminase activity and good heat stability which deserve further investigations on chicken liver L-asparaginase for its proper utilization. Also, the results showed that L-asparaginase has anti-proliferative activity in different cell lines growth *in vitro* (antitumor activity against hepatic and colon carcinoma).

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