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Purification and Characterization of L-glutaminase enzyme from camel liver: enzymatic anticancer property

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Abstract

L-Glutaminase has gained an important attention as glutamine-depleting enzyme in treatment of various cancers. Therefore, this study aimed to purify, characterize, investigate antitumor activity of L-glutaminase from camel liver mitochondria (CL-Glu), since no available information about CL-Glu from camel. CL-Glu was purified using cell fractionation, ultrafiltration, DEAE-and CM-cellulose chromatography columns. The purified CL-Glu was a monomer with a molecular weight of 70 ± 3 kDa, isoelectric point of 7.2, optimum temperature of 70°C and it was active over a broad pH range with a pH optimum at pH 8.0. Its activity had a clear dependence on phosphate ions. The studied enzyme showed sigmoidal kinetics, indicated its allosteric behavior with Km of 36 ± 4 mM and Hill coefficient of 1.5 which suggested a positive cooperatively of active sites. The purified L-glutaminase exerted antitumor activity against different cell lines with the highest cytotoxic activity against Hepatocellular carcinoma cell line (HepG-2) with an IC_{50} value of 152 µg/ml. In conclusion, L-glutaminase was purified from camel liver using simple methods and its unique properties such as stability at both wide pH range and at high temperature along with its relatively low molecular weight, facilitated its usage in medical applications as antitumor drug.

Keywords: Camel liver, Glutaminase, Purification, Characterization, Antitumor activity, Hepatocellular carcinoma.

1. Introduction

L-Glutaminase (L-Glutamine amidohydrolase E.C 3.5.1.2), a phosphate-activated enzyme that catalyzes the hydrolytic deamidation of L-glutamine by cleaving the side chain γ-amide bond to produce L-glutamate and ammonium ion **[1]**. It was localized to be loosely bound to the inner mitochondrial membranes of hepatocytes **[2]**, Also, glutaminases were distributed in all three domains of life including eukaryotes and prokaryotes **[3]**.

Conversion of glutamine to glutamate was the first and rate-limiting step of the glutaminolysis pathway in mitochondria and was catalyzed by L-glutaminase **[4]**. Glutaminolysis enables mammalian tissues to metabolize L-glutamine, hence exploiting its hydrolysis products **[5],** that participate in vital physiological processes such as; nucleotides and protein biosynthesis, neutralization of the reactive oxygen species (ROS) **[6]**, energy generation **[7]**, lipogenesis **[8]**, synthesis of neurotransmitters **[9]**, and blood pH buffering **[10]**.

L-Glutaminase was considered an essential enzyme for the synthesis of various nitrogen metabolic intermediates as a result of its substrate, L-glutamine was the most abundant amino acid in human body tissues **[11]**, thus providing a ready precursor for synthesis of macromolecules after being converted to L-glutamate **[12]**. Moreover, L-glutaminase had an important medical applications as anti-malignant and anti- retroviral treatment either alone or as combination therapy. Among its potential therapeutic uses are as an anti-cancer **[13]**, anti-leukemia **[14],** anti-HIV **[15].**

The Arabian one-humped camel (*Camelus dromedarius*) was one of the most common domestic mammals in Egypt, Northern Africa, and Arabian Peninsula which possesses a unique ecological and behavioral adaptations **[16]**. Besides, no data about this enzyme from camel liver was previously reported. So, the present study was designed to establish an economical and reproducible scheme for isolation, purification and characterization of L-glutaminase from camel liver mitochondria and evaluate the anti-tumor activity of the purified enzyme on different cancer cell lines.

2. Materials and methods

2.1. Enzyme source

Liver tissue samples of newly slaughtered adult male Arabian one-humped camel (*Camelus dromedaries*) were obtained from Cairo Slaughterhouse. Liver samples were obtained within an hour from sacrificing the animals. Visible fat, ligaments and connective tissue were trimmed and then washed

with cold isotonic saline solution (0.9 % NaCl) to remove contaminating erythrocytes until the drained washing saline is completely clear.

2.2. Mammalian cell lines

Human breast adenocarcinoma (MCF–7), human hepatocarcinoma (HepG–2) and human colorectal carcinoma (HCT–116) cell lines were obtained from the VACSERA (Giza, Egypt) tissue culture unit. The cancer cells lines were dispersed in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum, 1 % L-glutamine, HEPES buffer and 50 µg/ml gentamycin. All cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and were subcultured two times a week. For cytotoxicity assay, the cells were seeded in 96-wells, flat-bottomed Microtiter plate (Falcon, NJ, USA) at a cell concentration of 1 x 104 cells per well in 100 µl of growth medium. A Fresh medium containing different concentrations (two-fold serial dilutions) of the purified camel liver L-glutaminase was added to the confluent cell monolayers dispensed into the 96-wells after 24 hours of seeding.

2.3. Reagents and chemicals

Crystalline bovine serum albumin (BSA), DEAE–cellulose, CM-Cellulose (microgranular form and pre-swollen), Native and SDS standard molecular weight protein markers and isoelectric focusing protein marker kit were purchased from Sigma-Aldrich. St. Louis, USA. Sucrose and digitonin were purchased from Merck (Darmstadt, F.R.G.). L-glutamine and Nessler's reagent were obtained from Fisher scientific. All other chemicals used in this study were of analytical grade of the highest quality commercially available. Ammonia-free distilled water was used throughout the work.

2.4. Buffer solutions

Buffers were prepared according to Gomori **[17]** and pHs were checked using microprocessor bench-top pH meter Hanna model HI8417.

2.5. Assay of L-glutaminase activity

L-glutaminase activity was routinely assayed according to the method described by Imada et al. **[18]** with some modifications. The rate of L-glutamine hydrolysis was measured by spectrophotometric assay of the rate of ammonia liberated under the enzyme action using the direct nesslerization method.

L-glutaminase assay reaction mixture (2 ml) containing 40 mM L-glutamine and 50 mM potassium phosphate buffer (pH 8.0) was initiated by adding an appropriate volume of the enzyme solution to the reaction mixture. The assay reaction mixture was incubated for 15 at 37 ˚C min, then the reaction was arrested by adding 1.5 M TCA. Blank tubes were run by adding the enzyme preparation after the addition of trichloroacetic acid. The denatured proteins were removed by centrifugation and the liberated ammonia was determined in the supernatant by adding 0.2 ml of Nessler's reagent to 0.01 ml of the supernatant and the volume completed to 4 ml with ammonia-free distilled water. The optical density of the solution was measured against reagent blank at 450 nm after 1 min incubation. All enzyme assays were demonstrated to be linear with time and with protein concentration under the conditions employed. The ammonia standard curve was constructed using ammonium sulphate under the same assay conditions adopted for the sample to cover the range from 0.05 to 1 μmol/ml. The L-glutaminase unit was expressed as the protein concentration, which liberates one micromole of ammonia in one minute under standard assay conditions.

2.6. Protein quantification

The total protein concentration was quantified according to Lowry et al. **[19]** using bovine serum albumin (BSA) as a standard protein to cover the range from 20 – 200 μg/ml or according to Layne **[20]** by measuring ultraviolet light absorption at 280 nm.

2.7. Purification of mitochondrial camel liver L-glutaminase

2.7.1. Preparation of crude enzyme extract

Isolation of camel liver mitochondria was carried out according to the method of Heini et al. **[21]**. Ten grams of liver tissue were minced and mechanically homogenized on ice, with ten volumes (10 ml/g tissue) of the mitochondria isolation buffer (IB_m: 250 mM sucrose solution containing 1 mM EDTA). The liver homogenate was then centrifuged at 600×*g* for 15 min to remove tissue debris. The supernatant was then centrifuged at 9,000×*g* for 30 min to sediment the mitochondria. The mitochondrial pellets were washed once with IB_m and centrifuged again at 9,000×*g* for 30 min. The mitochondrial pellets were suspended in IB_m with the addition of digitonin (0.3 mg/ml) and the mixture was incubated for 15 min, centrifuged at 9,000 ×*g* for 30 min and the pellets of mitoplast were washed twice with IB_m and finally resuspended in the mitoplast suspension buffer (SB_m: 50 mM potassium phosphate buffer, pH 8.0 containing 10 % ethylene glycol and 10 μM leupeptin), then the mixture was

sonified for 45 seconds. The sonified suspension was centrifuged at 37,000 ×*g* for 30 min. The clear supernatant containing most of the enzyme activity was saved as crude enzyme extract and the pellet was discarded.

2.7.2. Membrane ultrafiltration

The crude enzyme extract was concentrated using pressurized Millipore Amicon stirred ultrafiltration cell with YM10 membrane whose molecular weight cutoff value equals 30 kDa, since proteins above the membrane molecular weight cut-off (30 kDa) was retained within the cell, while water and proteins below the cutoff value pass into the filtrate out of the cell. The obtained protein solution was designated as the concentrated ultrafiltrate.

2.7.3. Chromatography on diethylaminoethyl (DEAE) cellulose anion exchange column

The concentrated ultrafiltrate was chromatographed on DEAE-cellulose column previously equilibrated with 50 mM, pH 8.0 potassium phosphate buffer containing 1 mM EDTA and 10 % ethylene glycol (equilibration buffer). After the column was washed with the equilibration buffer, the adsorbed proteins were eluted using stepwise sodium chloride gradient ranging from 0.0–1.0 M in the equilibration buffer at a flow rate of 30 ml/h. Protein elution was tracked by measuring the absorbance at 280 nm and the enzyme activity was assayed in the collected fractions. Fractions containing L-glutaminase activities were pooled (DEAE-cellulose pooled fractions) and dialyzed overnight at 4 °C against 50 mM, pH 5.8 potassium phosphate buffer containing 1 mM EDTA and 10 % ethylene glycol (dialysis buffer), with three changes of the dialysis buffer.

2.7.4. Chromatography on carboxymethyl (CM) cellulose cation exchange column

The dialyzed DEAE-cellulose pooled fractions was mounted on top of CM-cellulose column previously equilibrated with 50 mM, pH 5.8 potassium phosphate buffer containing 1 mM EDTA and 10% ethylene glycol. After washing the column with the dialysis buffer, the adsorbed proteins were eluted using stepwise sodium chloride gradient ranging from 0.0–1.0 M in the dialysis buffer at a flow rate of 30 ml/h. Protein elution was tracked by measuring the absorbance at 280 nm and the enzyme activity was assayed in the collected fractions. Fractions containing L-glutaminase activity were pooled (CM-cellulose pooled fractions) and dialyzed overnight at 4 °C against the equilibration buffer with three buffer changes.

2.8. Electrophoretic analysis

2.8.1. Molecular weight determination

Native-polyacrylamide gel electrophoresis was carried out using 10 % PAGE according to Davis **[6]**. SDS-polyacrylamide gel electrophoresis was carried out using 15 % PAGE according to Laemmli [33]. Calibration curves were constructed between the molecular weights of the standard protein markers versus their relative mobilities (R_f), from which the molecular weight of the purified camel liver mitochondrial L-glutaminase was determined.

2.8.2. Isoelectric point determination

The isoelectric point (p*I*) of the purified camel liver mitochondrial L-glutaminase was determined according to the method of Giulian et al. **[22]** using 5 % native-polyacrylamide gel. Protein bands were visualized by staining with 0.025 % Coomassie Brilliant blue R-250.

2.9. Evaluation of the antiproliferative activity

The cytocidal effect of the purified camel liver L-glutaminase on three human cancer cell lines; HepG-2 liver cancer cell line, MCF-7 breast cancer cell line and HCT-116 colon cancer cell line were assessed by using crystal violet (CV) stain cell viability assay according to methods of Mosmann **[23]** and Gomha et al. **[24].**

3. Results

3.1. Purification of L-glutaminase

The specific activity of the camel liver crude enzyme extract was found to be 1.7 units/mg protein. 87.2 % of L-glutaminase was recovered in the concentrated ultrafiltrate solution with a specific activity of 2.3 units/mg protein and an increased fold purification of 1.4 over the crude enzyme extract. The first chromatography step on DEAE-cellulose column indicated that, L-glutaminase was adsorbed on DEAE-cellulose and eluted as a single peak by 0.1 M NaCl leading to an increase in the specific activity up to 47.9 units/mg protein with 28.2 fold purification, **Fig.1**. The pooled DEAE-cellulose fractions were dialyzed and applied on CM-cellulose column. The obtained elution profile of camel liver L-glutaminase on the CM-cellulose column revealed the presence of one sharp peak of L-glutaminase

activity as the enzyme is adsorbed on the CM-cellulose column, **Fig. 2**. L-glutaminase active fractions were pooled and dialyzed to give a highly purified final enzyme preparation with a final specific activity of 120 units/mg protein and 70.6 fold purification over the crude enzyme extract with a 53% yield. Results of L-glutaminase purification from camel liver mitochondria were summarized in **Table 1**.

3.2. Purity and homogeneity

Polyacrylamide slab gel electrophoresis was carried out for samples from different purification steps with sodium dodecyl sulfate (SDS) and β-mercaptoethanol in all gel preparations (denatured conditions). It can be seen from Fig. 3, that the dialyzed CM-pooled fractions appeared as a single band free from protein contamination indicating that camel liver L-glutaminase was purified to apparent homogeneity.

3.3. Electrophoretic analyses of camel liver L-glutaminase

The native-PAGE and SDS-PAGE showed that, the purified L-glutaminase gave only one distinctive band on both gels as illustrated in **Fig. 4 a& b**. This indicated that the purified enzyme was a protomer consists of a single protein chain. The apparent molecular weight was calculated to be 70 ± 3.5 kDa in reference to the calibration curves of standard protein markers. The purified camel liver L-glutaminase was electrofocused and the isoelectric point (p*I*) value was deduced to be 7.2, **Fig.4c**.

3.4. Michaelis-Menten constant (Km) and maximum velocity (Vmax)

Effect of substrate concentration on the velocity of purified L-glutaminase reaction was studied using a set of L-glutamine concentrations (10 - 100 mM). The results showed that as the glutamine concentration was increased, the enzyme activity increased from 4.70 to 24.26 units/ml at 100 mM L-glutamine, **Fig. 5a**. The enzyme activity showed substrate concentration dependence between 10 and 90 mM. The studied enzyme showed sigmoidal kinetics (S-shape plot), indicating its allosteric behavior. The Km and Vmax values for the purified camel liver L-glutaminase were calculated from the double reciprocal Lineweaver-Burk plot to be 36 ± 4 mM L-glutamine and 33 U/ml respectively, **Fig. 5b**.

3.5. Hill coefficient (n)

The Hill plot describes the fraction of the macromolecule saturated by a certain ligand as a function of the ligand concentration. By calculating Hill coefficient for the purified camel liver L-

glutaminase from the slope of Hill plot, a Hill coefficient value (n) equals 1.5 for the purified enzyme was obtained, **Fig. 5c**.

3.6. Effect of temperature

The effect of temperature on the reaction velocity was studied by using different incubation temperatures (4 – 85 °C). Figure 6 showed an increase in the activity of the purified enzyme as the temperature increased was observed and the optimum temperature of enzyme was at 70 °C with a significant change starting from 20 °C with continuation up to 70 °C, followed by a sharp decreased in the enzyme activity above 70 °C was observed which was completely lost at 85 °C.

3.7. Effect of pH

The effect of pH on the rate of the purified L-glutaminase-catalyzed reaction was studied in the pH range (4.0 - 10.0) using 50 mM potassium phosphate buffer to cover the mentioned pH range. The extremely acidic pH 4.0 and the extremely basic pH 10.0 phosphate buffer were reached by aid of 1 M HCl and 1 M NaOH, respectively. The standard enzyme assay procedure was applied in various pH media. It can be seen from **Figure7**, that the enzyme showed nearly the same reaction rate at a wide range of pHs (from 5.8 to 7.7) followed by an increase at pH 8.0. A sharp decrease in the rate of enzyme reaction at pHs above 8.0 was observed, indicated that a pH 8.0 was the optimum pH of camel liver L-glutaminase. When using the acetate buffer, which covered the acidic pH range (3.6 – 5.6) and Tris-HCl buffer which covered the alkaline pH range (7.2 – 9.0), the rate of the enzyme reaction was very low. This uncovers the fact that, the nature of the buffer made a significant differencein the pH curve even within the overlapping pH ranges covered by different buffering systems. This was due to the phosphate-dependant nature of the enzyme, which manifested as undetectable L-glutaminase activity while using any buffer system other than the phosphate one.

3.8. Effect of some non-substrate ligands

The effect of various group specific chemicals (EDTA as metal chelator, β-ME as reducing agent and PMSF as a serine protease inhibitor), ions (ammonium as NH_4Cl and, phosphate as Na_3PO_4) and amino acids (DL-leucine, DL-isoleucine, L-glutamate, L-aspartate and DL-asparagine), on the purified L-glutaminase activity was studied using L-glutamine as substrate in the presence or absence of L-

glutaminase **(Tables 2, 3&4)**. A control test without any investigated substance was considered as 100 % residual activity. The results showed that, PMSF, EDTA, β-ME, L-glutamate and L-aspartate were of no effect on the enzyme activity. An increase in L-glutaminase activity was observed upon increasing concentrations of either phosphate or ammonium ions, hence, these ions wereconsidered as activators, whereas L-leucine, L-isoleucine and DL-asparagine inhibited the enzyme.

3.9. Inhibition studies

To investigate the type of inhibition displayed by L-leucine, L-isoleucine and DL-asparagine as potential inhibitors using L-glutamine as substrate, the enzyme activity was assayed in the absence and presence of varying concentrations of these inhibitors. Lineweaver-Burk plots data indicated that DL-asparagine had a competitive inhibitory effect while DL-leucine and DL-isoleucine exerted a potent non-competitive inhibition, **Fig. 8 a, b& c**, respectively. Also, the present results showed that DL-asparagine (Asn) was the most potent inhibitor followed be DL-leucine (Leu) and DL-isoleucine (Ile), was the weakest one as deduced from the IC_{50} values. The values of IC_{50} and the type of inhibition for each amino acid were tabulated in Table 5.

3.10. Antitumor activity

The cytotoxic activity of L-glutaminase was evaluated against three mammalian cancer cell lines including Human Hepatocellular carcinoma (HepG-2), Human colon carcinoma (HCT-116) and Human breast cancer cell line (MCF-7) using crystal violet at different concentrations of the purified enzyme. Results showed the potential antitumor activity of CL-Glu with the highest cytotoxic activity against HepG-2, **Fig. 9a** and HCT-116, **Fig. 9a** (IC_{50} 152 and 183 µg/ml, respectively). While, lower cytotoxic activity exerted on MCF-7 as indicated from IC_{50} = 219 µg/ml, **Fig. 9c**.

4. Discussion

The one of the cancer treatment difficulties is the high toxicities of chemotherapeutic drugs toward normal cells. Many tumors depend on the extracellular pool of one or more amino acids to fulfill protein biosynthesis demands due to their inability to synthesize them. Therefore, the use of enzymatic treatment that cause the systemic depletion of one of tumor-essential amino acids, result in tumor apoptosis with minimal side effects to normal cells. In addition, the use of enzyme from animal source

was better than that of microbial origin in medical applications, due to reduce the immunogenicity toward the animal enzyme. Thus, the demand for amino acid-depleting enzyme with unique properties from animal source for the treatment of cancer remains high [25]. So, The present study was designed to set-up a simple and reproducible methods for purification of L-glutaminase from camel liver then studying its molecular and kinetic properties concomitant with evaluation ofits anti-tumor activity *in vitro*.

In the present study, L-glutaminase appeared to be purified to homogeneity about 70-fold over the crude enzyme extract with final specific activity of 120 units/mg proteins and 53% recovery using ultrafiltration membrane, DEAE-cellulose anion exchange chromatography and CM- cellulose cation exchange chromatography. Enzyme stabilization was achieved by 10 μM leupeptin, 1 mM EDTA and 10 % ethylene glycol. Heini et al. **[21]** purified rat liver L-glutaminase with specific activity (31.6 units/mg proteins), 395 purification fold and 0.6 % yield using ammonium sulphate precipitation, DEAE-Sephacel ion exchange chromatography and hydroxyapatite chromatography, along with digitonin, chymostatin or leupeptin, and ethylene glycol in preparation of mitochondria to stabilize the enzyme. Yeast L-glutaminase from *Debaromyces sp*. was purified by a protocol that involved protamine sulphate treatment, Biosep-DEAE-P weak anion exchange column, gel filtration on Sephacryl S-200 HR and the biocompatible (titanium) 1050 Hewlett-Packard strong anion exchange liquid chromatography gave specific activity of 10917 units/mg proteins, 2607 purification fold and 2.4 % recovery **[26]**.

In the current study, the enzyme was completely adsorbed on the anion exchange resin and was eluted as a single peak by 0.1 M NaCl in equilibration buffer, pH 8.0. This chromatographic behavior of the concentrated mitochondrial crude enzyme solution of DEAE – cellulose revealed that at pH 8.0, the enzyme has a high net negative charge as shown by its high affinity for the anion exchange resin. This behavioral observation was in agreement with previous results reported for L-glutaminase purified from rat liver mitochondria **[21, 27]**.

The purified camel liver L-glutaminase was found to consist of single protein chain with molecular mass of 70 ± 3.5 kDa as determined by both native-PAGE and denaturing SDS-PAGE. Hepatic L-glutaminases were reported to be 63 kDa for the purified recombinant mature human L-glutaminase **[28],** 58 kDa in mouse **[29]** and 58 - 59.2 kDa in rat **[30]**.

Variations in estimation of molecular weight values of rat liver L-type glutaminase were previously reported; it was reported to be the homotrimeric protein with native molecular weight approximately 172.5 ± 10 kDa using sucrose gradient ultracentrifugation **[21, 31]**. While, it was reported

to be homotetrameric protein with native molecular weight approximately 290 - 310 kDa using size exclusion chromatography on Sephacryl S-300 **[27]**.

A reasonable clarification of this distinction was that, the produced protein by recombinant DNA technology may somehow differs from the native protein processing in mammalian cell systems as reported by Campos-Sandoval et al**. [28].**

In the present study, determination of isoelectric point (P*I*) of the purified mitochondrial camel liver L-glutaminase was found to equal 7.2. This value complies with the enzyme adsorption and elution behavior on both DEAE-cellulose and CM-cellulose chromatography columns. Isoelectric point of camel liver L-glutaminase was close to that of pig brain glutaminase, which was 6.7 **[31].**

The effect of temperature on the enzyme activity was determined in the range from 4 to 85 °C using the standard enzyme assay. The obtained current results showed that camel liver L-glutaminase has an optimum temperature at 70 °C, reflecting the thermostable nature of the enzyme purified from camel liver. This thermostable nature was previously reported for other camel liver enzymes such as camel liver glutathione-S-transferase **[32],** L-asparaginase **[33]** and camel liver L-arginase **[34]**. With respect to mammalian glutaminases, little data were reported about the heat-tolerant nature of the enzyme. The maleate-activated L-glutaminase isozyme isolated from rat kidney had heat resistance up to 50 ºC [35]. Also, L-glutaminase from *Penicillium brevicompactum* NRC 829 retained 92 % of its initial activity after incubation at 70 ºC for 30 min indicated the thermostable nature of this enzyme **[36].**

The changes of the purified camel liver L-glutaminase activity in different buffers (phosphate, acetate and Tris-HCl) with respect to variation of pH were investigated. In the present study, there was an increase in L-glutaminase activity of camel liver in the pH range of 4.0 – 5.8, followed by a relatively constant activity over a broader pH range between pH 6.0 and 8.0 with a pH optimum at 8.0, using phosphate buffer. Outside these pH ranges, the activity decreased rapidly. However, no detectable L-glutaminase activity was observed. Whereas, using acetate and Tris-HCl buffers as compared to phosphate buffer revealing the phosphate-dependence nature of the purified L-glutaminase. These results were in agreement with that reported in human liver **[37]** and rat liver L-glutaminases **[21, 27, 37]**. The maximum activity of the partially purified rat liver glutaminase was virtually constant between pH 7.7 and 8.5, but it decreased strongly below pH 7.6 **[27, 35]**.

The kinetic studies of the purified camel liver L-glutaminase exhibit a non-Michaelis-Menten kinetics with a clear sigmoidal dependence of the rate of glutamine hydrolysis on substrate concentration, indicating the allosteric regulation of the enzyme with low affinity for L-glutamine

as expected for most of mammalian L-type enzymes**[39, 40].** This kinetic behavior was in agreement with that reported for the purified recombinant human L-type enzyme **[28]**, human liver **[37]**, mouse liver and the sonicated rat liver mitochondrial L-glutaminases **[40].**

The importance of the allosteric regulation of L-glutaminase activity appears as it functions as a regulator of ureagenesis, since the product of L-glutamine hydrolysis; L-glutamate, is the precursor for N-acetylglutamate, which is needed for carbamoyl synthase. Beside this, the produced ammonia, which is also generated by glutaminase- mediated glutamine hydrolysis is also a substrate for carbamoyl phosphate synthesis. Hence, the activity of L-glutaminase plays an important role in the regulation of hepatic urea synthesis and its allosteric regulation enables the fast elimination of excess glutamine *via* the urea cycle **[39, 40].**

The Km value for camel liver L-glutaminase for the main physiological substrate L-glutamine was calculated to be 36 ± 4 mM. The reported Km values were 20 mM for phospholipase A2 treated frozen and thawed rat liver mitochondria **[41]**, 21 ± 1.4 mM for sonicated rat liver mitochondrial L-glutaminase **[2, 27]**, 28 mM for soluble rat liver L-glutaminase **[35]** and 32 mM for recombinant human L-type glutaminase **[27]**.

Camel liver L-glutaminase gave a linear response with a Hill coefficient value of 1.5, suggested the presence of two or more binding sites for the substrate, L-glutamine on the purified enzyme molecule. This result indicated the positive cooperativity with respect to substrate binding, which was advantageous for such enzyme that known to serve as a control point in metabolism providing, a mechanism for regulation of enzyme activity [42]

Similar results of Hill coefficient (greater than 1.0) were previously reported using L glutamine concentration range between 5 and 50 mM, and phosphate buffer concentration of 100 mM (pH 8.0), Hill plots for glutaminases from human liver mitochondria and rat liver mitochondria gave Hill coefficient values of 1.9 and 1.8, respectively **[37]**.

In the current work, studying the effect of some non- substrate substances on the activity of the purified enzyme revealed that, CL-Glu activity did not affected by EDTA, PMSF and β-ME as examples of some organic substances. The same observation was previously reported for the effect of EDTA on mammalian L-glutaminase **[43,44]** which indicated that L-glutaminase was not a metalloenzyme. **Durá et al. [45]** reported no inhibitory effect exerted by PMSF (a serine protease inhibitor) on glutaminase from *Debaryomyces spp.* This finding suggests that the hydroxyl groups of serine or threonine residues are not involved in the binding of the substrate to the L-type

enzymes active site and that it does not belong to the classical serine hydrolases family as an authentic amidohydrolase.

The CL-Glu was neither inhibited nor activated by the reducing agent β-Mercaptoethanol, the same as *Penicillium brevicompactum* NRC 829 L-glutaminase **[37]**. This finding indicated that, the sulfhydryl group(s) –SH may not participate in the active site of the enzyme.

In the current study, upon investigation of the ammonium ion effect on the activity of the reaction catalyzed by glutaminase, it was observed that, ammonium ion has a slight activating effect on the purified enzyme under the adopted assay conditions (pH 8.0). In agreement with the present data, Patel and McGivan **[28]** reported that, ammonium chloride had no apparent activating effect at pH 8.0. Also, human L-type glutaminase was reported to be slightly activated by ammonia **[29].** Also, the bicarbonate anion (HCO_3^-) appeared to have no apparent effect on purified CL-Glu activity, which in agreement with the pig renal glutaminase behavior **[46]**.

In the present study, the purified camel liver L-glutaminase activity increased by increasing phosphate concentration. This behavior was complied with the data from previous studies, has found that an activity was completely dependent on the added phosphate for rat liver glutaminase **[21, 27, 35, 47]**. Also, the same result was reported for human Wild-type glutaminase C **[12].** It has been reported that the activity of membrane bound glutaminase was independent of the added phosphate while the activity of the solubilized enzyme preparation was completely phosphate dependent [2, 40]**.** Since in the absence of phosphate, a very low rate of glutamine hydrolysis was observed, thus phosphate was considered as an obligatory activator of liver glutaminase **[37, 44, 48].**

The effect of some amino acids on the activity of the purified camel liver L- glutaminase was also examined. When studying the effect of glutamine and asparagine acid-analogues, respectively, on the enzyme activity, it was found that, L-glutamate and L-aspartate had no considerable interference with the activity as expected, due to the absence of the side chain amide group, upon which L-glutaminase act on. While, DL-asparagine exerted a competitive inhibition toward camel liver L-glutaminase. This observation was in contrast with the non-competitive inhibition of L-asparagine on glutaminase from the cell-free extract of *Debaryomyces spp.* **[45]**. By compilation of the results of both DL-asparagine and L-aspartic acid, it can be concluded that the –NH2 moiety of the amide group of asparagine seems to be the one responsible for the inhibition, since L-aspartate did not exert any effect on the enzyme activity.

Based on the current results, it was concluded that camel liver L-glutaminases agreed with the Pseudomonas 7A glutaminase-asparaginase enzyme preparation in being not inhibited by L-aspartate **[43]** and it was not inhibited by its product, L-glutamate as a result of enzyme activity, a well known characteristic of L-type glutaminases **[43]**. Previous studies with the partially purified rat liver enzyme **[27]**, purified rat liver enzyme **[21]** and intact mitochondria isolated from human liver **[37]** confirmed that liver glutaminase was not inhibited by its product L-glutamate.

In the current study, the effect of the branched-chain amino acids, leucine and isoleucine on the activity of L-glutaminase showed that, both of them were non- competitive inhibitors. In the review literature inspected, leucine (up to 1 mM), and other branched-chain amino acids, have been shown to stimulate glutaminases in intact rat hepatocytes **[49]**. However, leucine (up to 5 mM) has no activating influence on the activity of the partially purified rat liver L-glutaminase **[27]**

The cytotoxic activity of the purified camel liver enzyme was conducted to evaluate its cytocidal effect against various cancer cell lines *in vitro* as a promising and potential treatment for various tumors in the future. Accordingly, the cytotoxic effects and the biological activity of L-glutaminase as an antitumor agent were examined *in vitro* against HepG-2, MCF-7 and HCT-116 using crystal violet cytotoxicity bioassay.

The present results showed the potent effect of the purified CL-Glu in a dose dependent manner, where increasing the activity of the applied pure L-glutaminase – as milligram protein resulted in an increased percentage of dead cells. The highest cytotoxic activity (IC_{50}) was recorded towards human liver carcinoma cell line HepG-2, which in agreement with those reported for *Penicillium brevicompactum NRC* 829L-glutaminase **[36]** and *Streptomyces canarius* **[50]**. However, the least cytotoxic activity was obtained towards the breast cancer cell line MCF-7 with IC_{50} = 219 µg/ml. This value greatly coincides with the IC_{50} values of 250 µg/ml and 283.288 µg/ml reported for L-glutaminases isolated from *Aspergillus flavus* KUGF009 **[51]** and *Aspergillus oryzae* **[52]**, respectively. Meanwhile, Aly et al. **[53]** reported a low IC_{50} value of 10 µg/ml exhibited by L-glutaminase isolated from *Streptomyces sp.* D214 against MCF-7 cancer cell line. While, Reda **[50]** reported that the growth of MCF-7 cells was not affected by *Streptomyces canarius* L-glutaminase. The moderate cell growth inhibitory effect exerted by the pure mitochondrial camel liver L-glutaminase was against the human colon cancer cell line HCT-116, compatible with the results reported for *Streptomyces canarius* L- glutaminase **[50].**

Most neoplasms are said to be "glutamine addicted" **[12, 54]** because most tumor cells have an absolute requirement for glutamine as a growth substrate **[51]**. The absolute requirement for glutamine as a component of tissue culture media for many mammalian cells suggested the use of glutaminase as a carcinostatic tool in depleting the glutamine needed for tumor growth. Thus depriving the cancerous cell from such metabolically important amino acid by reducing its blood concentrations to undetectable levels **[55, 56]**. The requirement of glutamine for the synthesis of nucleic acids suggested that the depletion of glutamine through the action of glutaminase would result in an altered nucleic acid metabolism of the treated tumor cells through the depression of both de novo nucleotide synthesis and utilization of preformed adenine for such synthesis by the glutaminase-treated cells **[57].**

In conclusion, the results of the present study indicated that mitochondrial camel liver L-glutaminase share some of its molecular and biochemical properties with either mammalian or microbial glutaminases. Indeed, It was found that, the purified L-glutaminase possesses some desirable properties which qualifies it for its testing as a potential cancer treatment. The enzyme was considered to be therapeutically suitable due to: its high stability at physiologic pH range; it didn't require cofactors; its high substrate specificity; it wasn't inhibited by the products of the reaction which it catalyzed; it consisted of a single polypeptide chain, which was favorable for being insusceptible to dissociation into its constituting subunits during manipulation; its relatively low molecular weight, which minimize the elicitation of an immune response; and promising antiproliferative activity against different cell lines *in vitro*, especially the human hepatocellular carcinoma cell line (HepG-2).

Conflict of interests

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Table 1

Purification steps of camel liver L-glutaminase.

Steps of purification	Total activity (Unit)*	Total protein (mg)	Specific activity (U/mg)**	Fold Purification	Yield (%)
1. Crude extract	190.4	110.6	1.7	1.0	100

2.	Ultrafiltration	166	73	2.3	1.4	87.2
3.	DEAE-cellulose pooled fractions	158	3.3	47.9	28.2	82.9
4.	Dialyzed DEAE pooled fractions	147	3.3	44.5	26.2	77.2
5.	CM-cellulose pooled fractions	113.2	0.84	134.7	79.3	59.4
6.	Dialyzed CM-Pooled fractions	100.8	0.84	120	70.6	53

*L-glutaminase unit was expressed as the protein concentration which liberates one micromole (µmol) of ammonia in one minute under standard assay conditions.

**Specific activity was expressed as units per milligram protein.

Table 2

Effect of organic non-substrate ligands on the activity of camel liver mitochondrial L-glutaminase.

Organic substance	Working concentration (mM)	Remaining activity (%)
Phenylmethylsulfonyl	0.2	89

fluoride (PMSF)	0.5	96
	1	93
β-Mercaptoethanol (β-ME)	0.2	88
	0.5	88
	1	95
Ethylenediaminetetraacetic acid (EDTA)	0.5	100
	1	101
	5	96

*****Values are the means of triplicate determinations

Table 3

Effect of amino acids (non-substrate ligands) on the activity of camel liver mitochondrial L-glutaminase.

Amino acid	Working concentration	Remaining activity

	(mM)	(%)
DL-Asparagine (Asn)	10	68
	20	16
	25	15
	40	14.5
	50	14
DL-Leucine (Leu)	0.02	24
	0.025	21
	0.05	18
	0.075	14
	0.1	11
DL-Isoleucine (Ile)	0.02	70
	0.025	54
	0.05	41
	0.075	27
	0.1	18
L-Aspartic acid (Asp)	10	90
	20	92
	50	96
L-Glutamic acid (Glu)	10	86
	20	97
	50	88

*Values are the means of triplicate determinations.

Table 4

Effect of ions (non-substrate ligands) on the activity of camel liver mitochondrial L-glutaminase.

Ions	**Working concentration (mM)**	**Enzyme activity (%)**
Phosphate ion (Na_3PO_4)	10	75
	20	95
	50	107
Ammonium ion (NH_4Cl)	1	95
	2	98
	5	136

*Values are the means of triplicate determinations.

Table 5

Type of inhibition and IC_{50} values for the non-substrate ligands upon enzyme activity.

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Non-substrate ligands	Type of inhibition	IC_{50} Experimental value (mM)
DL-Asparagine	Competitive	15.6
DL-Leucine	Non-competitive	0.013
DL-Isoleucine	Non-competitive	0.027

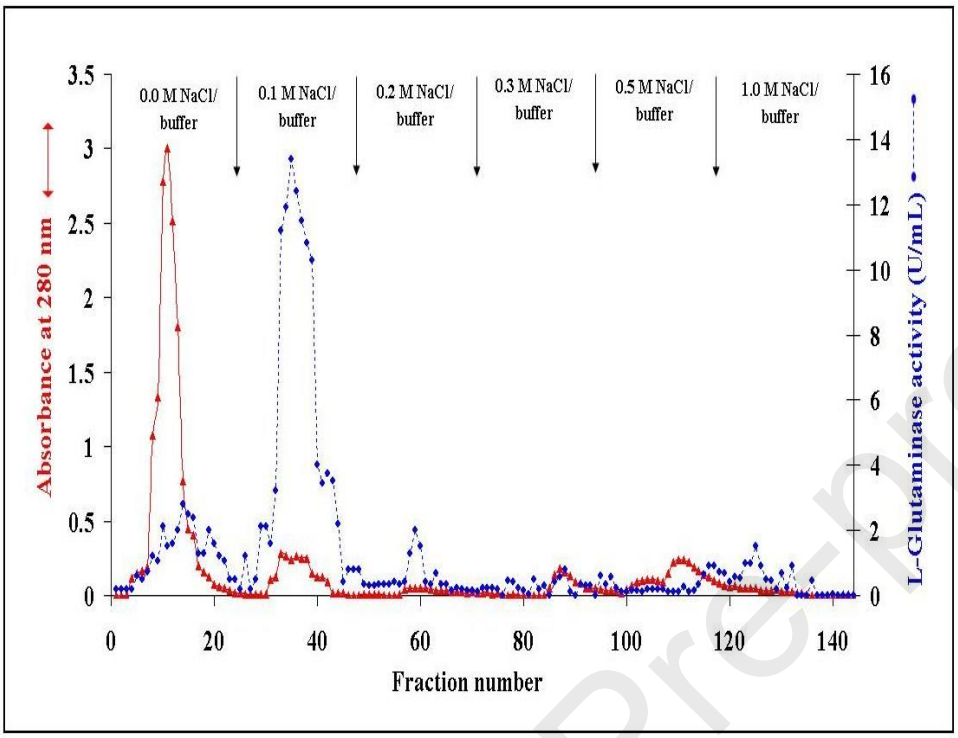

Fig. 1. DEAE-cellulose column chromatography of concentrated enzyme solution. A stepwise gradient of 0.0 - 1.0 M NaCl in equilibration buffer was used to elute fractions 1 - 144 at a flow rate 30 mL/hour and 2 mL/fraction.

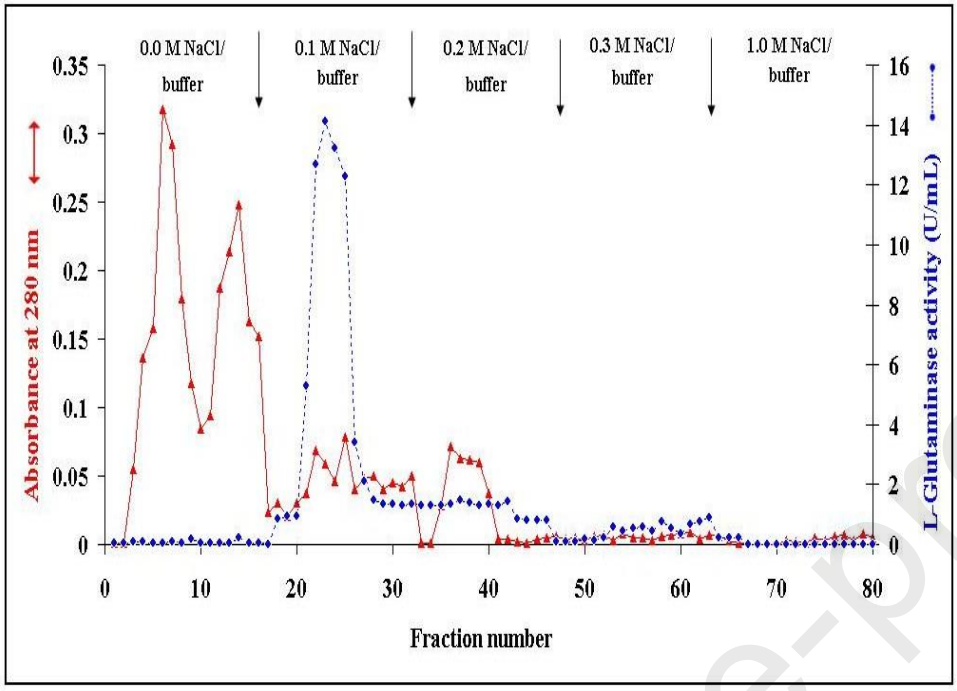

Fig. 2. CM-cellulose column chromatography of dialyzed DEAE-enzyme solution. A stepwise gradient of 0.0-1.0 M NaCl in dialysis buffer was used to elute fractions 1-80 at a flow rate 30 ml/hour and 2 ml/fraction.

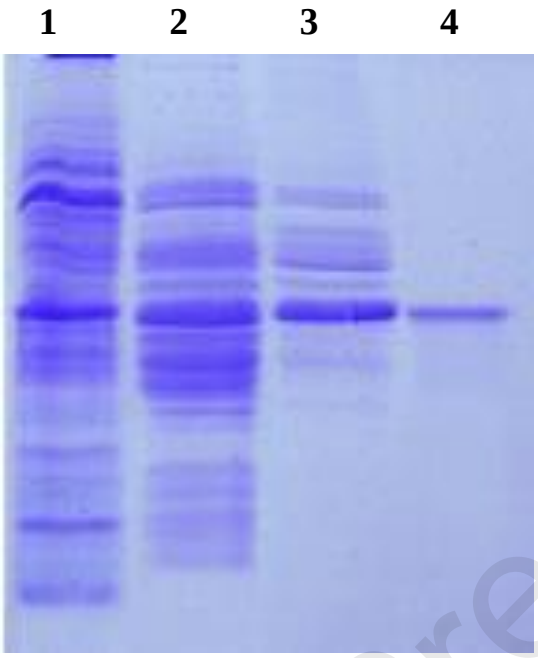

Fig. 3. SDS-polyacrylamide gel electrophoresis (15%) pattern of camel liver L-glutaminase samples from different purification steps. The following samples were applied; (1) Crude extract (2) Ultrafiltration sample (3) Dialyzed DEAE-pooled fractions (4) Dialyzed CM-pooled fractions. The gel was stained with Coomassie Brilliant blue R-250 stain.

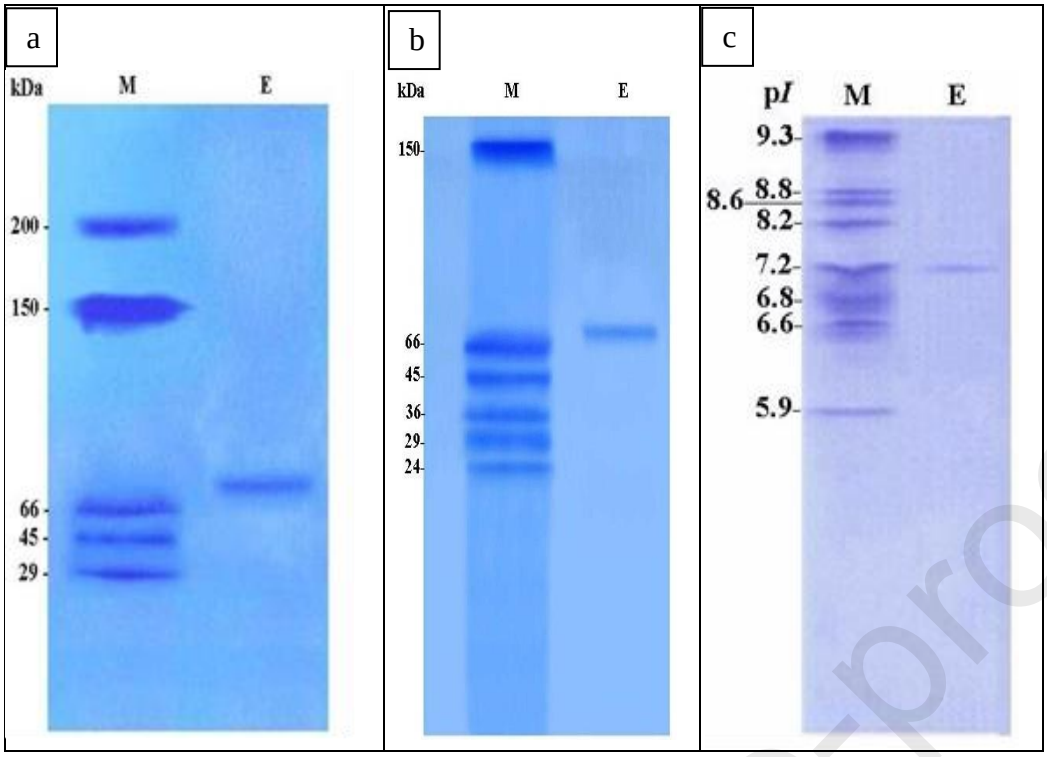

Fig. 4. Electrophoretic analysis of the purified camel liver mitochondrial L-glutaminase (E) on **a)** Native polyacrylamide gel electrophoresis, **b)** SDS-polyacrylamide gel elecrophoresis and **c)** Native isoelectric focusing referring to standard markers (M). The gel was stained with Coomassie Brilliant blue R-250 stain.

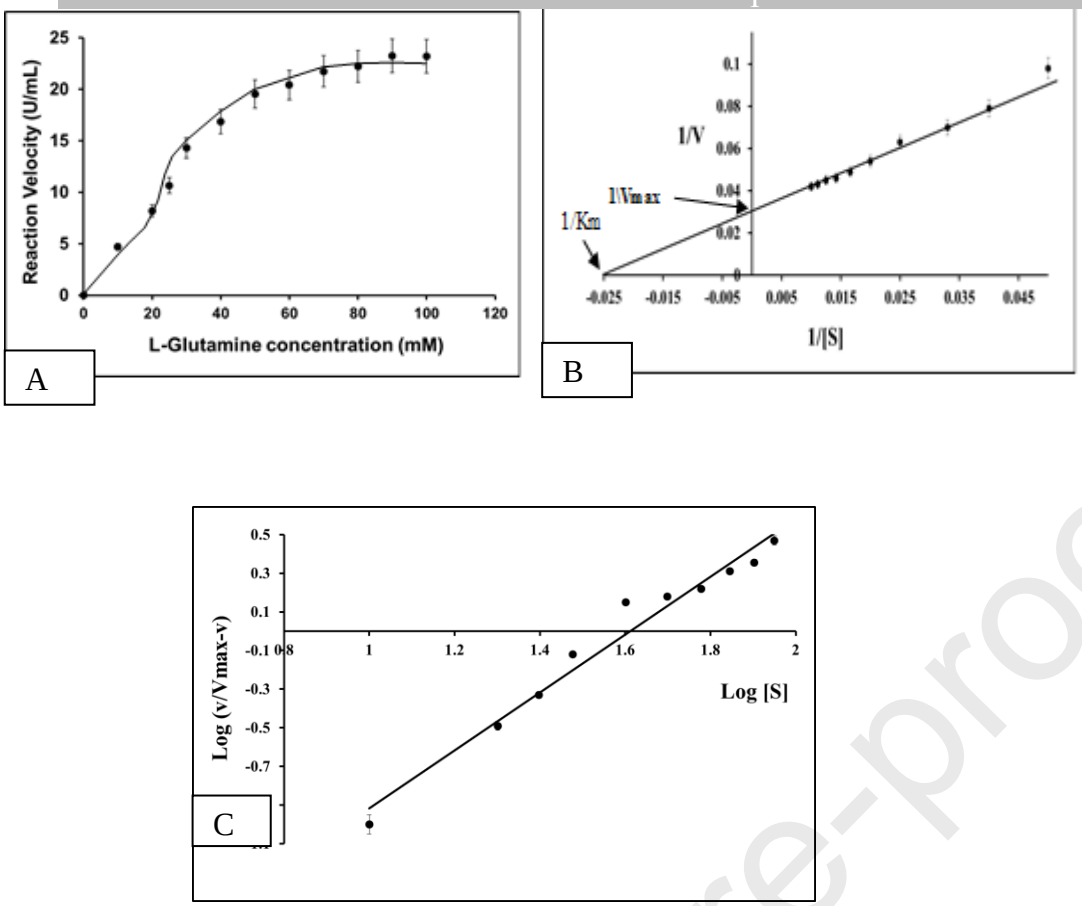

Fig. 5. L-glutaminase kinetic parameters **(a)** Effect of L-glutamine concentration on the velocity of camel liver mitochondrial L-glutaminase. **(b)** Lineweaver-Burk plot. **(c)** Hill plot. Values are means of triplicate determinations.

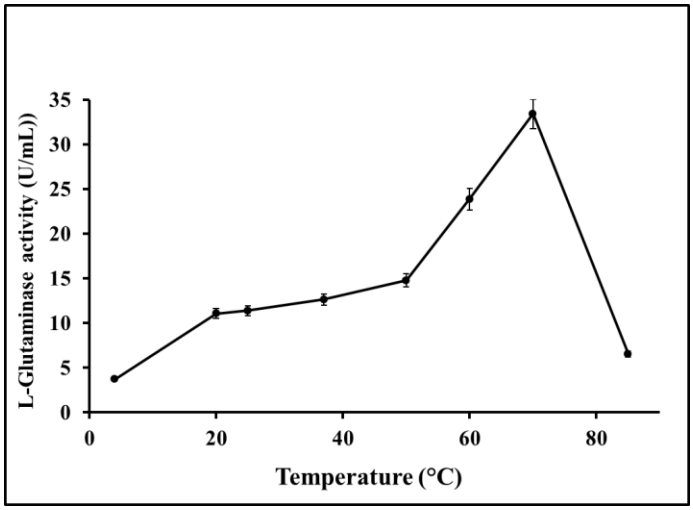

Fig. 6. Effect of temperature on the activity of camel liver mitochondrial L-glutaminase. Values are means of triplicate determinations.

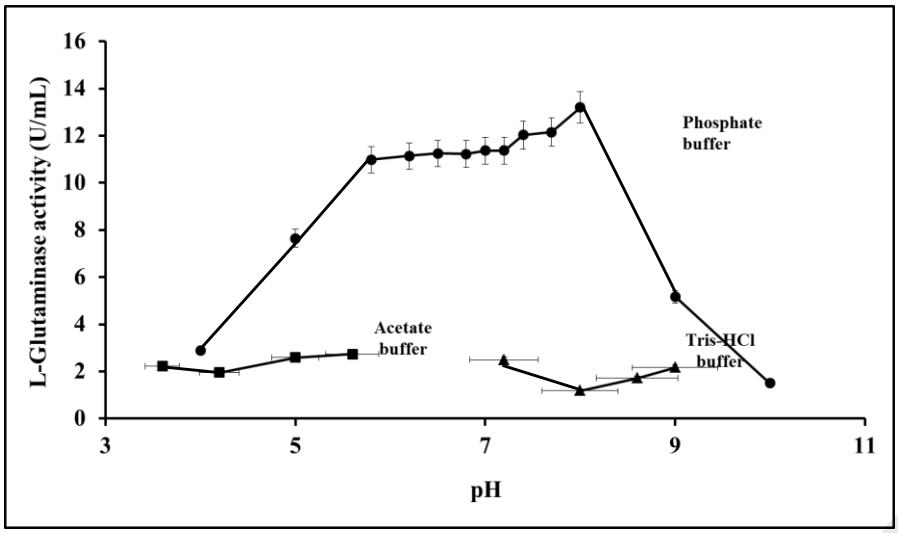

Fig. 7. Effect of pH on the activity of camel liver mitochondrial L-glutaminase. Values are means of triplicate determinations.

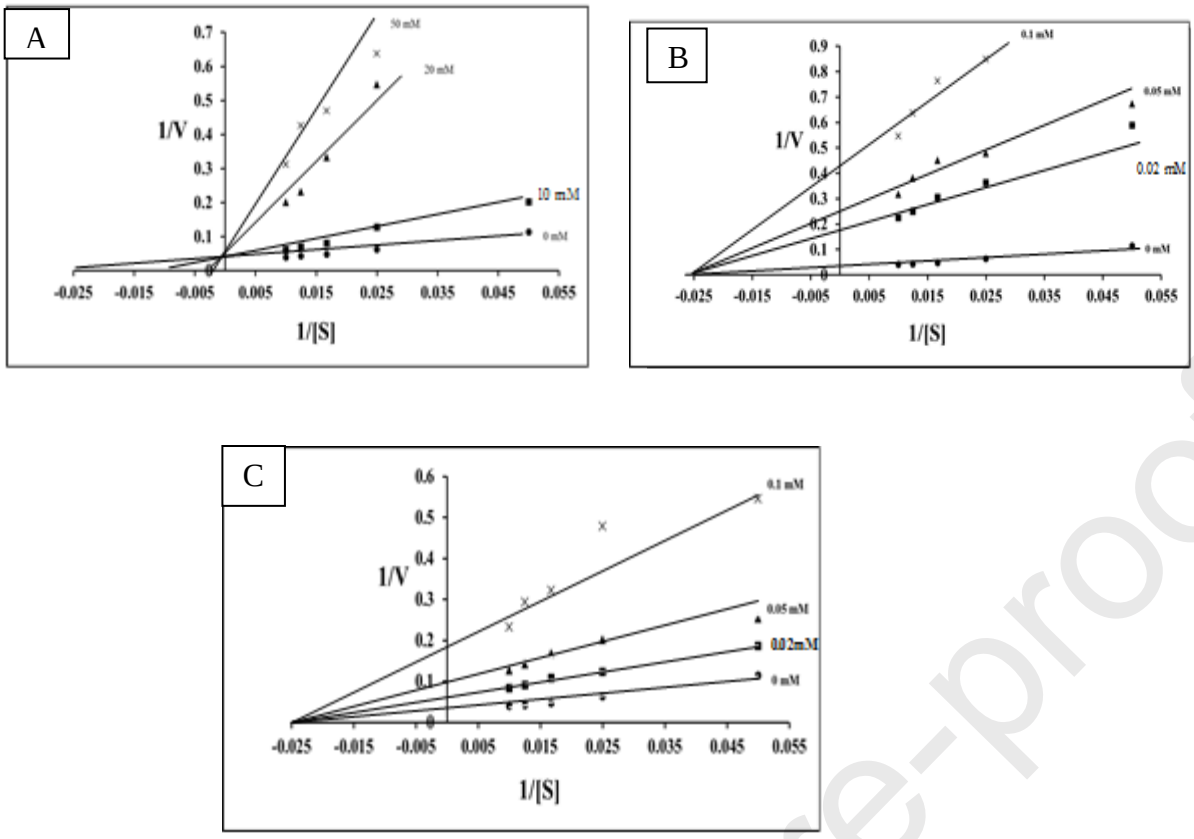

Fig. 8. Lineweaver-Burk plot relating purified camel liver L-glutaminase reaction velocity to L-glutamine concentration in absence and presence of different concentrations of **(a)** DL-asparagine (10, 20, 50 mM), **(b)** DL-leucine (0.02, 0.05, 0.1 mM) and **(c)** DL-isoleucine (0.02, 0.05, 0.1 mM). Values are means of triplicate determinations.

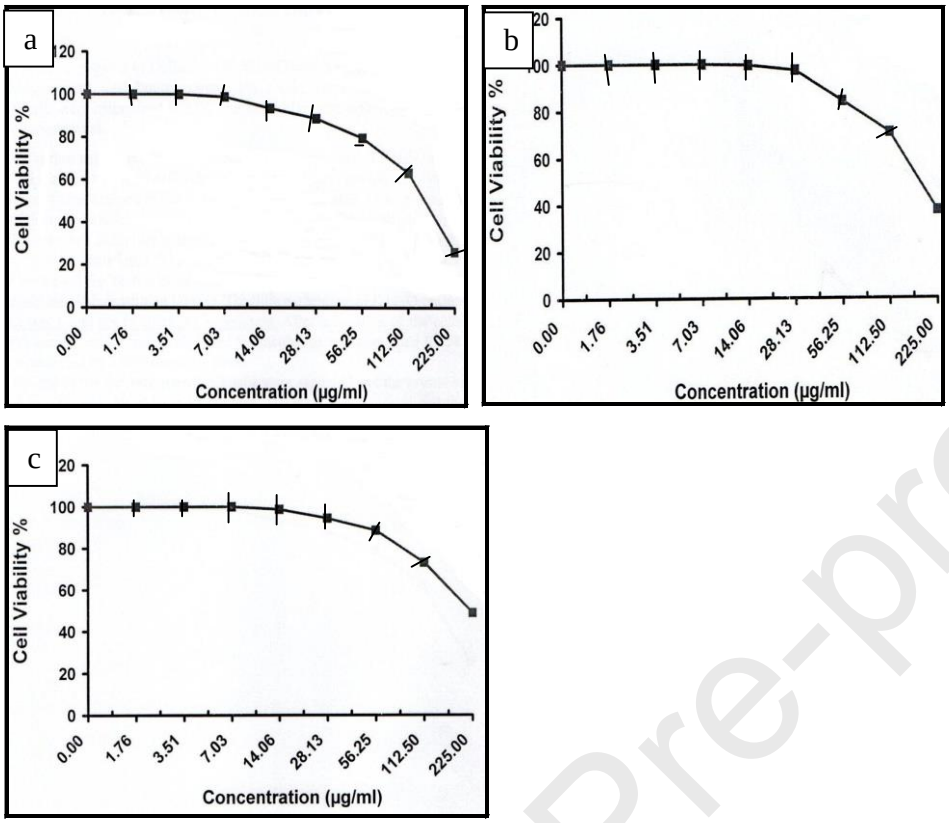

Fig. 9. Cytotoxic activity of mitochondrial camel liver L-glutaminase against **(a)** HepG-2, **(b)** HCT-116 and **(c)** MCF-7 cell lines. Values are means of triplicate determinations.