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Purification, Kinetic Properties and Antitumor Activity of L-Glutaminase from *Penicillium brevicompactum* NRC 829

Ali Mohamed Elshafei[1*], Mohamed Mohamed Hassan[1], Nadia Hussein Ali[1], Mohamed Abd-Elmontasr Abouzeid[2], Dalia Ali Mahmoud[2] and Dina Helmy Elghonemy[1]

[1]Department of Microbial Chemistry, National Research Centre, Dokki, Cairo, Egypt.
[2]Microbiology Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author AME designed the study, all authors performed the statistical analysis, wrote the protocol, author DHE wrote the first draft of the manuscript, managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: The aims of the present study were to purify and characterize L-glutaminase from *Penicillium brevicompactum* NRC 829; and to evaluate the antitumor activity of the purified enzyme against different tumor human cell lines.
Study Design: Testing of antitumor activity of L-glutaminase, purified from a filamentous fungal strain, against four different tumor human cell lines.
Place and Duration of Study: Department of Microbial Chemistry, Genetic Engineering and Biotechnology Division, National Research Centre (NRC), Cairo, Egypt, between January 2011 and February 2012.
Methodology: *P. brevicompactum* NRC 829 was grown and maintained on modified Czapek Dox agar (MCD) medium. Cell-free extract was directly used as the source of crude enzyme. L-glutaminase was purified by heat treatment for 20 min at 50ºC, followed by gel filtration on Sephadex G-100 and G-200 columns.
Results: An intracellular L-glutaminase from *Penicillium brevicompactum* NRC 829 was purified to homogeneity (162.75 fold) with an apparent molecular mass (M_r) of 71 kDa. The purified enzyme showed its maximal activity against L-glutamine when incubated at pH 8.5

**Corresponding author: Email: alielshafei@yahoo.com;*

at 50ºC for 30 min. The purified enzyme retained about 92 % of its initial activity after incubation at 70ºC for 30 min indicating the thermo-stability nature of this enzyme. The highest activity was reported towards its natural substrate, L-glutamine, with an apparent K_m value of 1.66 mM. The purified enzyme inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2), with IC_{50} value of 63.3µg/ml.
Conclusion: L-glutaminase purified from *Penicillium brevicompactum* NRC 829 is a potential candidate in food and pharmaceutical industries.

Keywords: L-glutaminase; Penicillium brevicompactum NRC 829; purification; characterization; antitumor activity.

1. INTRODUCTION

L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is an industrially important hydrolytic enzyme catalyzes the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Fig. 1) [1]. Glutamic acid is known to be an important amino acid contributing not only to the pleasant taste “Umami”, but also to the nutritional properties of food. Therefore, the addition of safe starter cultures containing glutaminase activity to fermented sausages is desirable because this enzyme is able to act on L-glutamine, present in relatively high amounts in the fresh mix, generating ammonia, as a neutralizer of acidity, and L-glutamate, as a flavour enhancer [2].

In recent years L-glutaminase has attracted much attention with respect to its wide applications in pharmaceuticals as an anti-leukemic agent [3,4], and also as an efficient anti-retroviral agent, along with its use for the production of specialty chemicals like theanine by c-glutamyl transfer reactions [5]. Another important application of L-glutaminase is as biosensoring for glutamine levels monitoring in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid [6]. Furthermore, the enzyme regulates the cerebral concentrations of glutamine and glutamate, which are very important in processes such as ammonia detoxification [7].

L-glutaminase plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes [8]. Among different sources of L-glutaminase enzyme producers, microbial strains possess an elevated edge over other enzyme producers due to their growth requirements, easy processing and handling….etc [9]. L-glutaminase is ubiquitous in a large number of microorganisms including bacteria, moulds and yeasts [10,11]. Since the present source for this enzyme is limited to *Escherichia coli*, *Erwinia cartovora* and *Aspergillus oryzae*, a search for potential strains that hyper produces this enzyme with novel properties under economically viable bioprocesses is pursued [12]. This led to the continuous screening program for isolation of novel microbial strains that could produce an effective enzyme with few limitations at usage sectors [13].

Thammarongtham et al. [14] purified L-glutaminase from *Aspergillus oryzae* by using ammonium sulfate precipitation (45-75%), then applied to Q-Sepharose ion exchange column and finally chromatographed on Sephacryl S-200 HR gel filtration column. The molecular weight of the purified glutaminase on SDS-PAGE was estimated to be about 90 kDa. L-glutaminase was purified from the cell-free extract of *Debaryomyces* sp. CECT 11815 by protamine sulphate treatment and several chromatographic procedures including anion exchange chromatography and gel filtration. The purified enzyme consisted of two subunits, with molecular masses of 65 and 50 kDa, respectively. Activity was optimal at 40ºC and pH 8.5, and the K_m value for L-glutamine was 4.5 mM [2].

In a previous publication, we explained the various optimization procedures for the improvement of L-glutaminase production in submerged state fermentation [15]. The present study deals with the purification and characterization of L-glutaminase from *Penicillium brevicompactum* NRC 829; the work will be extended to evaluate the antitumor activity of the purified enzyme against different tumor human cell lines.

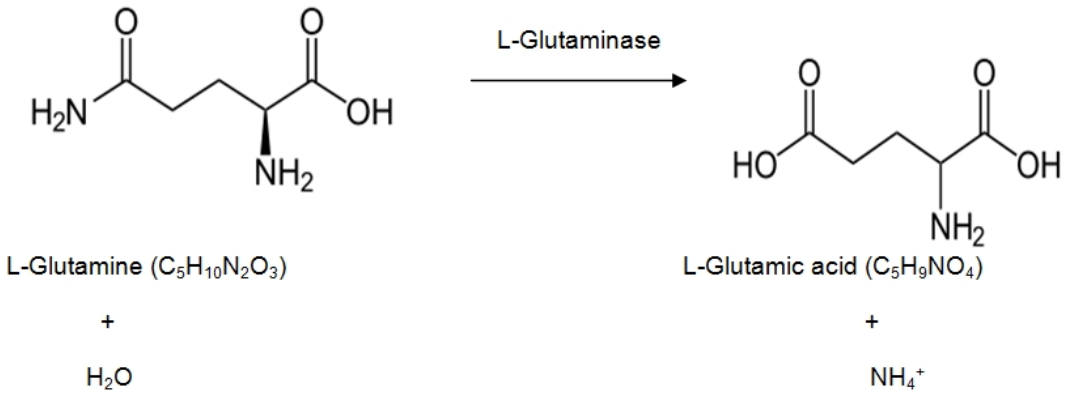

Fig. 1. Schematic representation of mechanism of action of L- glutaminase

2. MATERIALS AND METHODS

2.1 Microorganism

Penicillium brevicompactum NRC 829, a local isolated strain was obtained from the Culture Collection of the Microbial Chemistry Department, National Research Centre of Egypt. The organism was grown and maintained by weekly transfer on slants of modified Czapek Dox agar (MCD) medium [16] supplemented with 2% D-glucose as the only carbon source for growth.

2.2 Chemicals and Buffers

Anhydrous L-glutamine, trichloroacetic acid, Nessler`s reagent chemicals, bovine serum albumin and reagents for electrophoresis were obtained from Sigma chemical CO. (St Louis, Mo). Sephadex G-100 and Sephadex G-200 for chromatography were obtained from Pharmacia Fine Chemicals (Sweden). Molecular weight markers for SDS-polyacrylamide gel electrophoresis were purchased from Fermentas Company; U.S.A. RPMI 1640 medium was purchased from Lonza Company, Belgium. MTT salt medium was purchased from BioBasic Company, Canada. All other chemicals were of the best analytical grade and of high purity. Buffers were prepared according to Gomori [17].

2.3 Growth Conditions for Fungal Culture

P. brevicompactum NRC 829 was grown on modified Czapek Dox agar slants containing (g/L): glucose, 20.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5, and agar 20.0. The pH of the medium was adjusted to 5.0 and slants were incubated at 28ºC for 7 days. After incubation, conidia were scraped and 5.0 ml of sterile distilled water was added to slant

and the spores were extracted by hand-shaking. Then, 2.0 ml aliquots were used to inoculate 250 ml Erlenmeyer flasks, each containing 50 ml of sterilized modified Czapek Dox's broth medium. Thereafter, the inoculated flasks were incubated for 4 days at 28ºC under static condition.

2.4 Preparation of Cell-Free Extracts

The cultures were harvested by filtration and the mycelial mats were rinsed thoroughly with sterile ice cold distilled water, and blotted dry with absorbent paper. The fresh fungal biomass was thoroughly ground with approximately twice its weight of sterile washed cold sand in a chilled mortar [18]. The cell contents were extracted with cold 0.1M boric acid borate buffer pH 8.0, thereafter, the slurry obtained was centrifuged at 5500 rpm for 15 min at 4ºC and the supernatant was directly used as the source of enzyme.

2.5 Enzyme Assay

L-glutaminase enzyme assay was performed using a colorimetric method by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent. For routine assay 0.1 ml of properly diluted enzyme was added to 0.4 ml of 0.025 M L-glutamine solution in 0.1 M boric acid borate buffer (pH 8.0). Incubated for 30 min at 37ºC and the reaction was stopped by the addition of 0.5 ml of 1N H_2SO_4. The precipitated protein was removed by centrifugation and 0.2 ml of supernatant was added to 3.8 ml of distilled water. Thereafter, 0.5 ml of Nessler`s reagent was added, and the absorbance was measured at 400 nm within 1 to 3 min. Enzyme and substrate blanks were included in all assays, and a standard curve was prepared with ammonium chloride, the enzyme activity was expressed as unit (U)/ml [19]. One unit of L-glutaminase is defined as the amount of enzyme that liberates one micromole (µmol) of ammonia per minute under the standard conditions. The specific activity (sp. activity) is defined as the units of L-glutaminase per milligram protein [20].

2.6 Determination of Protein Concentration

Protein content in the crude enzyme preparation was determined according to Bradford [21] using bovine serum albumin as the standard. Proteins in the purified fractions were monitored according to Schleif and Wensink [22].

2.7 Purification of L-glutaminase

2.7.1 Heat treatment

The crude enzyme extracts were heated at 50ºC for 20 min, the tube was immediately cooled in ice bath and the sediment formed was removed by cooling centrifugation at 5500 rpm (-4ºC) for 10 min [19].

2.7.2 Sephadex G-100 gel filtration

The most active partially purified enzyme fraction from the previous step was applied on a Sephadex G-100 column (1.5 x 50 cm) that was pre-equilibrated with a 0.05 M boric acid borate buffer pH 8.0 at a flow rate of 20 ml/hr. The fractions were collected and examined for enzyme activity and protein content. The most active fractions were pooled together,

dialyzed against the 0.01 M boric acid borate buffer (pH 8.0), and concentrated by lyophilization (-50ºC).

2.7.3 Sephadex G-200 gel filtration

The purified fraction obtained from the previous step was loaded onto the pre-equilibrated Sephadex G-200 column (2.0 x 50 cm) with 0.05 M boric acid borate buffer (pH 8.0), at a flow rate of 10 ml/h. The fractions were collected and examined for L-glutaminase activity and protein content. The most active fractions were pooled, concentrated by lyophilization and stored at -20ºC.

2.8 Molecular Weight Determination by SDS-PAGE

The polyacrylamide separating gel (main gel) (12%) and stacking gel (5%) were prepared according to Laemmli [23], The log molecular weight of different standard molecular weight marker proteins (260, 130, 95, 72, 55, 35 and 28 kDa) was plotted against their relative mobility in the gel for two hours. The gel was directly placed in Coomassie brilliant blue R-250 staining solution for two hrs, destained several times for two hrs, photographed while wet, dried and kept for comparison for calculation of M_r of the purified L-glutaminase.

2.9 Optimal Reaction Time

This experiment was carried out to identify the optimal incubation time for L-glutaminase activity by incubating the standard reaction mixtures in a period of time ranging from 5 to 120 min.

2.10 Effect of pH on Enzyme Activity and Stability

The activity of L-glutaminase was evaluated at different pH values. The purified enzyme was incubated using 0.1 M of four buffers, in the range between pH 3 - 10, under assay conditions and the amount of ammonia liberated was determined. Buffers used were citrate-phosphate (pH 3.0 - 7.0), sodium-phosphate (pH 6.0 - 8.0), boric acid borate (pH 8.0 - 9.0) and glycine-NaOH (9.0 - 10). In case of pH stability experiment, the enzyme was incubated for 24 hrs at 4 ± 1ºC at different pH values in the absence of substrate and the residual activity was determined.

2.11 Effect of Temperature

Optimum temperature for enzyme activity was determined by incubating the standard reaction mixture at temperatures ranging from 10 - 90ºC. Thermostability studies were carried out by pre-incubating the enzyme at different temperatures (50, 60, 70 and 80ºC) for different time intervals (5.0 - 60 min).

2.12 Substrate Specificity and Determination of K_m

Identical reaction mixtures containing the same amount of enzyme preparation were made, each received an equimolar amount (10 µmoles) of a specific substrate namely L-asparagine, L-glutamine, D-asparagine, D-glutamine, Nicotinamide Adenine Dinucleotide (NAD), acetamide, and acrylamide and incubated under the standard assay conditions. The Michaelis constant (K_m) value of the purified enzyme was estimated in a range of L-glutamine

concentrations of 0.05–30 µmoles. The apparent K_m value of the purified L-glutaminase was calculated from the Lineweaver-Burk plots relating 1 / V to 1 / [S] [24].

2.13 Effect of Different Metallic Salts and Various Compounds on Enzyme Activity

The effect of metal ions of several mineral salts (i.e. Na^+, K^+, Ba^{2+}, Hg^{2+}, Co^{2+}, Ca^{2+} and Cu^{2+}), EDTA (ethylenediamine-tetraacetate), iodoacetate, reduced glutathione and 2-mercaptoethanol on the enzyme activity was tested at different concentrations (10^{-3} M, 5 x 10^{-2} M and 10^{-2} M). After the exposure time (2hrs), enzyme activity in each sample was measured and expressed as a relative activity percentage calculated from the ratio of the activity of the treated L-glutaminase to that of the untreated sample.

2.14 Effect of Different Concentrations of NaCl

The enzyme activity was assayed separately in the standard reaction mixture supplemented with NaCl at various concentrations (0 – 30%) (w/v) and relative activity was expressed as the percentage in the absence of NaCl.

2.15 Antitumor Activity

The antiproliferative effect of the purified enzyme on different tumor human cell lines; Hep G 2 (Human hepatocellular carcinoma cell line), – MCF 7 (breast cancer cell line), HCT-116 (colon cell line) – A-549 (human lung Carcinoma]) was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) to purple formazan [25].

2.16 Statistical analysis

Statistical analysis was carried out according to the method described by Kenney and Keeping [26].

3. RESULTS AND DISCUSSION

3.1 Purification of L-glutaminase Enzyme from *Penicillium brevicompactum* NRC 829

The sequential multi-steps purification procedure was summarized in Table 1. Fig. 2 shows the elution profile of purification of the partial purified L-glutaminase on Sephadex G-100 column. The most active fractions (F24-F27) for enzyme activity with specific activity 191.90 U/ mg protein, purification fold of about 36 and 69% recovery yield were pooled together, dialyzed against 0.01 M boric acid borate buffer (pH 8.0), and concentrated by lyophilization (-50ºC).

The elution profile of the most active fractions collected from Sephadex G-100 and loaded on Sephadex G-200 column is illustrated in Fig. 3. A sharp distinctive peak of L-glutaminase activity, which fits with only one protein peak, was noticed. The most active fractions (F16-F18) with specific activity of 869.1 U/ mg and about 162 purification fold and 48% enzyme recovery were pooled together, concentrated with lyophilizer and stored at -20ºC.

Separation and characterization of L-glutaminases from *Cryptococcus albidus* and *C. albidus* (ATCC-20293) have been investigated by Iwasa et al. [27] and Ohshita et al. [28], respectively. Purification of L-glutaminase from cell-free extracts of *Debaryomyces* sp. CECT 11815 was achieved by protamine sulphate treatment followed by anion exchange chromatography and gel filtration as reported by Dura et al. [2].

3.2 Molecular Weight Determination by SDS–PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of the Sephadex G-200 column. It revealed only a single distinctive protein band for the pure preparation of L-glutaminase with an apparent molecular weight of 71 kDa (Fig. 4).

There has been wide variation in the molecular weight of L-glutaminases produced from different sources, which could range between 40 and 180 kDa. L-glutaminase with a higher molecular weight (148 kDa) was purified from *Ps. aurantiaca* by Imada et al. [29]. While 40 kDa molecular weight L-glutaminase by *Ps. nitroreducens* [30] is the lowest molecular weight recorded for amidase enzyme produced by microorganisms.

Most of the L-glutaminases are monomers; however, some of the bacterial glutaminases are reported as dimmers and tetramers. In this concern, Marine *Micrococcus luteus* K-3 constitutively produced two salt-tolerant glutaminases, designated glutaminase I and II with the same molecular weight of about 86 kDa [31].

3.3 Kinetic Properties of the Purified L-glutaminase

3.3.1 Effect of incubation time

Results obtained indicate that L-glutaminase activity (Fig. 5), increased as the incubation time increased up to 60 min, after which the linearity of the reaction was not presented and only a slight increase in amidase enzyme activity was reported.

3.3.2 Effect of pH on enzyme activity and stability

L-glutaminase is one of the amidases that are generally active and stable at neutral and alkaline pH. Results (Fig. 6) revealed that pH 8.5 was the optimal pH for L-glutaminase activity from *P. brevicompactum* using boric acid-borate buffer. In agreement with our results, Ohshima et al. [32] reported that, pH 7.0 to 9.0 to be the optimum pH for amidase activity. In this concern, an optimum pH range of 7.5 - 9.0 was found for L-glutaminase produced from *Pseudomonas aeruginosa* [19]. L-glutaminase from *Debaryomyces* sp. CECT 11815 and *Lactobacillus rhamnosus* showed an optimal working at pH of 8.5 and 7.0, respectively [2,33].

The purified L-glutaminase from *P. brevicompactum* NRC 829 was more stable in alkaline pH than the acidic one; it retains 100% activity at pH 8.5 even after incubation for 24 hrs at 4 ± 1ºC (Table 2). In addition, pH from 7.0 to 9.0 seems to be the most suitable pH range for the storage of this enzyme. While, a slight decrease in the amidase enzyme activity was observed when samples were stored at pH 9.5-10. Our results also demonstrated that, L-glutaminase retained about 56 % of its activity after storing at pH 4.0 for 24 hrs.

3.3.3 Effect of temperature and thermal stability behavior

As far as the temperature dependence of L-glutaminases activity is concerned, many of the L-glutaminases reported to have both optimal and stable temperature of around 40 to 50ºC. The purified L-glutaminase was active over a wide range of temperature from 30ºC to 75ºC with an optimum at 50ºC (Fig. 7). A notable decrease in enzyme activity was observed at higher temperatures above this value. At 70ºC about 73% of L-glutaminase activity was still present, while at 90ºC, it showed maximum loss of enzyme activity. L-glutaminase obtained from *Aspergillus oryzae* revealed optimum activity in a temperature range of 37 to 45ºC [34]. L-Glutamine was highly deamidated at 60ºC by glutamine amydohydrolase enzyme partially purified from *Penicillium politans* NRC 510 [35].

The results of temperature effect on enzyme stability indicated that no significant enzyme activity was lost when it was preincubated at 50°C to 60°C for 60 min (Fig. 8). L-glutaminase retained about 92 % of initial activity after incubation (in the absence of substrate) at 70ºC for 30 min. Moreover, L-glutaminase was still retaining about 66% of the original activity, after incubation at 80ºC for 5 min, which revealed the high thermal stability of L-glutaminase. These results indicate the thermophilic nature of the purified amidase enzyme produced by *P. brevicompactum* NRC 829.

In this concern, L-glutaminase purified from *Aspergillus oryzae* is stable up to 45ºC but lost its activity completely at 55ºC [34]. Prusiner et al. [36] performed the *E. coli* L-glutaminase stability studies at low temperatures and the authors observed that the exposure of enzyme to cold temperatures resulted in a reversible inactivation of enzymatic activity, while subsequent warming to 24ºC restored the activity and no protein denaturation occurred during this process.

3.3.4 Substrate specificity and K_m

The substrate specificity of the enzyme is presented in Table 3. The results revealed that among the different substrates tested, the highest apparent affinity of L-glutaminase was reported towards its natural substrate L-glutamine while the least activity was occurred with acetamide (Table 3). In this concern, Wakayama et al. [37] reported that L-glutaminase from *Stenotrophomonas maltophilia* NYW-81 hydrolyzed L-glutamine, L-asparagine, D-glutamine, and D-asparagine, however; the highest specific activity was reported towards L-glutamine.

Dura et al. [2] reported that L-glutaminase purified from *Debaryomyces* spp. CECT 11815 exhibited activity towards L-γ-Glu-methyl ester, L-γ-Glu-hydrazide, and L-albiziin, while L-asparagine, CBZ-L -Gln, CBZ-L -Gln-Gly, glutathione, L-γ-Glu-pNA and L-γ-Glu-AMC were not hydrolysed.

The K_m value of L-glutaminase for L-glutamine was found to be 1.66 mM (Fig. 9). This result indicates the high affinity of L- glutaminase towards its natural substrate, which might relate to its degree of effectiveness against tumors. Dura et al. [2] reported a higher K_m value of L-glutaminase purified from *Debaryomyces* spp. CECT 11815 for L-glutamine (4.5 mM). Weingand-Ziadé et al. [33] reported that the L-glutaminase activity purified from *Lactobacillus rhamnosus* showed typical Michaelis–Menten behavior with an affinity constant K_m of 4.8 mM for L-glutamine.

3.3.5 Effect of different metallic salts and various compounds

Among the salts tested, considerable loss of activity was observed only with Hg^{2+} and Cu^{2+} while Na^{+} or K^{+} acting somehow as an enhancer (Table 4). EDTA has no effect on enzyme activity which indicates that L-glutaminase might not be a metalloenzyme. L-glutaminase is neither inhibited nor activated by reducing agents compounds including 2-mercaptoethanol (2-ME) and reduced glutathione (GSH) or thiol group blocking (namely iodoacetate) which indicates the absence of evidence for the involvement of SH group(s) in the catalytic site of this enzyme.

3.3.6 Effect of different concentrations of NaCl

Results obtained in Fig. 10, indicate that, L-glutaminase activity increases gradually with the increase of NaCl concentrations up to 20 %.While, above this value, a gradual decrease in the enzyme activity took place. It is worth to mention that even in the presence of 30 % NaCl, L-glutaminase still retaining about 95 % of its activity. These results indicate that L-glutaminase from *P. brevicompactum* NRC 829 has significant advantages over other microbial L-glutaminases in the production of glutamic acid due to high salt tolerance behavior.

Salt-tolerant enzymes may play potentially significant roles in industrial processes that require high salt environments like the soy sauce fermentation. Thus, salt tolerant L-glutaminases were patented for use in industrial processes [38]. Moriguchi et al. [31]) have proposed the use of salt tolerant L-glutaminase from bacteria as a possible alternative, since their enzymes could be halophilic rather than halotolerant allowing the use of high salt concentrations.

High salt-tolerance of L-glutaminase produced by *Lactobacillus rhamnosus* was reported [2] [33], where the presence of 5% (w/v) salt increased L-glutaminase activity almost two-fold and 90% of the initial activity still remained at 15% (w/v) salt. On the other hand, L-glutaminases from other sources (*Aspergillus oryzae*) are markedly inhibited by high salt concentrations as demonstrated by Yano et al. [39].

3.3.7 Antitumor activity

Using MTT assay, the in vitro bioassay cytotoxic effect of *Penicillium brevicompactum* NRC 829 L-glutaminase on the growth of four human tumor cell lines namely Hep-G2 [Human hepatocellular carcinoma cell line], MCF-7 [Breast cancer cell line], HCT-116 [Colon cell line] and A549 [Human lung Carcinoma] showed that the crude-enzyme extracts have anti-proliferative activity in different cell lines growth (Table 5). However, the highest antitumor activity was recorded towards Hep-G2 (65.3%), while the least activity was obtained towards A-549 (33%) when compared with the growth of untreated control cells.

Therefore, Hep-G2 cell line was selected for further evaluation using partial purified and pure enzyme. The incubation of Hep-G2 with gradual doses of *Penicillium brevicompactum* NRC 829 L-glutaminase (partially purified and purified enzyme) lead to a gradual inhibition in the cell growth as concluded from the low IC_{50} values of 109.9 and 63.3 µg/ml, respectively (Table 6). In this connection, the cytotoxicity of L-glutaminase from *Aspergillus flavus* KUGF009 towards MCF-7 cell lines by the MTT assay (IC_{50} 250 µg/ml) was reported by Nathiya et al. [40].

Table 1. Sequential multi-steps process for purification of L-glutaminase from *P. brevicompactum* NRC 829

Purification step	Total activity (U)	Total protein (mg)	Sp. activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	667± 0.34	125 ± 0.43	5.34	100.0	1.0
F2 (Heating at 50°C for 20 min)	624.5 ± 0.16	25.12 ± 0.18	24.86	93.63	4.66
Sephadex G-100 L-Glutaminase (F24-F27)	456.7 ± 0.23	2.38 ± 0.31	191.90	68.47	35.94
Sephadex G-200 L-Glutaminase (F16-F18)	321.6 ± 0.37	0.37 ± 0.29	869.08	48.21	162.75

The volume of the culture from which the intracellular crude-enzyme extracts obtained was two litres. Data is expressed as mean ± S.D. of triplicates.

Table 2. Determination of pH stability of purified L-glutaminase

Buffer (0.05 M)	pH value	Relative activity (%)
Control		100
Citrate-phosphate	3.0	50
	4.0	56
	5.0	65
	6.0	73
	7.0	90
Sodium-phosphate	6.0	76
	7.0	91
	8.0	97
Boric-acid borate	8.0	99
	8.5	100
	9.0	98
Glycine-NaOH	9.0	96
	9.5	89
	10.0	80

Reaction mixture contained: L-glutamine, 10µmoles; boric-acid borate, 40 µmoles; extract protein, 22 µg; total vol. 0.5 ml; at pH, 8.5; 50°C; for 30 min.

Table 3. Substrate specificity of purified L-glutaminase

Substrate (10 µmoles)	Relative activity (%)
L-Asparagine	37
L-Glutamine	100
D-Asparagine	18.0
D-Glutamine	00.0
NAD	00.0
Acetamide	07.0
Acrylamide	63.0

Reaction mixture contained: Substrate, 10µmoles; boric-acid borate buffer, 40 µmoles; extract protein, 22 µg; total vol. 0.5 ml; at pH, 8.5; 50°C; for 30 min.

Table 4. Effect of different metal cations and various compounds on L-glutaminase activity

Activator or inhibitor	Relative activity (%)		
	10^{-3}M	5 x 10^{-2}M	10^{-2} M
Control	100	100	100
NaCl	110	127	143
KCl	103	124	139
$BaCl_2$	104	98	93
$HgCl_2$	70	49	37
$CoCl_2$	112	105	101
$CaCl_2$	104	98	94
$CuCl_2$	72	56	44
EDTA	99	99	97
Iodoacetate	100	99	99
R-Glutathione	101	101	98
2-Mercaptoethanol	103	100	98

Reaction mixture contained: L-glutamine, 10 µmoles; boric-acid borate buffer, 40 µmoles, extract protein, 22 µg; compound, as indicated; total vol. 0.5 ml; at pH 8.5; 50°C; for 30 min.

Table 5. Anti-tumor activity of crude L-glutaminase from *Penicillium brevicompactum* NRC 829 on the growth of four human tumor cell lines

Sample	Hep-G 2	Hct-116	MCF-7	A-549
Crude L-glutaminase	65.3%	41%	34.2%	33%

Table 6. Anti-tumor activity of partially purified and purified L-glutaminase on the growth of Hep-G2

Sample	IC_{50} (µg/ml)	IC_{90} (µg/ml)	Remarks
Purified L-glutaminase	63.3	114.7	72.4% at100µg/ml
Partially purified L-glutaminase	109.9	180.5	38.4% at100µg/ml

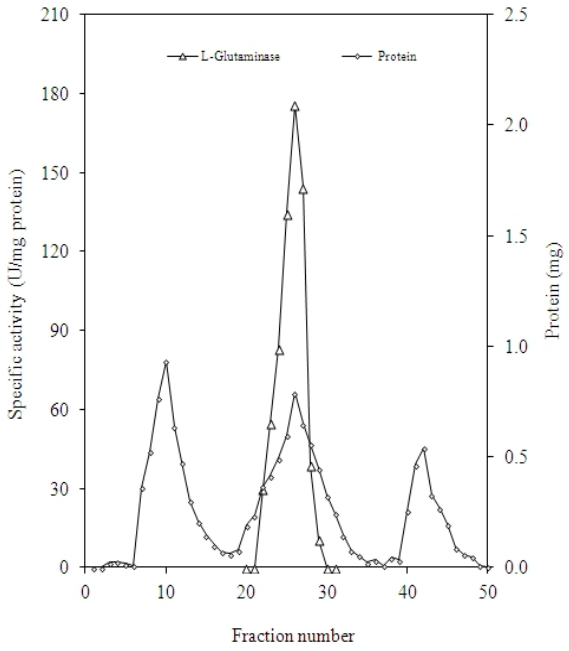

Fig. 2. Elution diagram of L-glutaminase of *P. brevicompactum* from Sephadex G-100

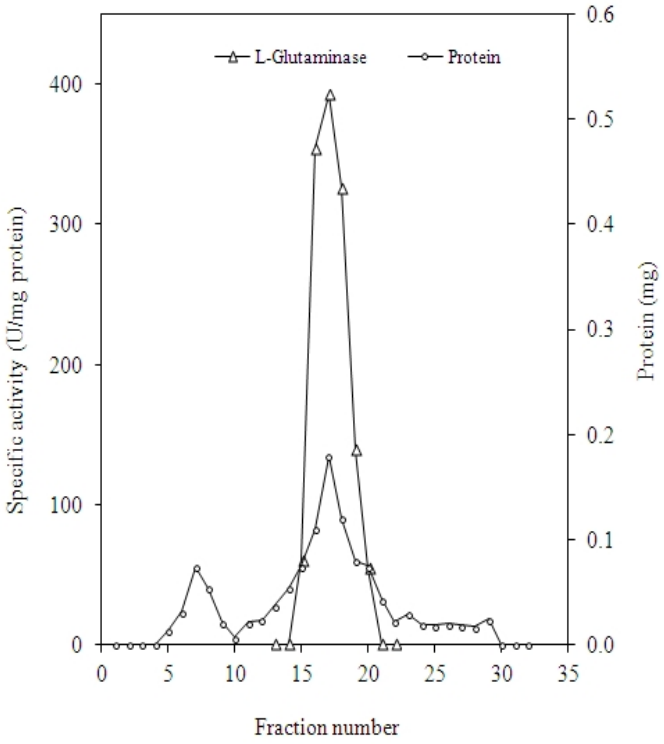

Fig. 3. Elution diagram of L-glutaminase of *P. brevicompactum* from Sephadex G-200

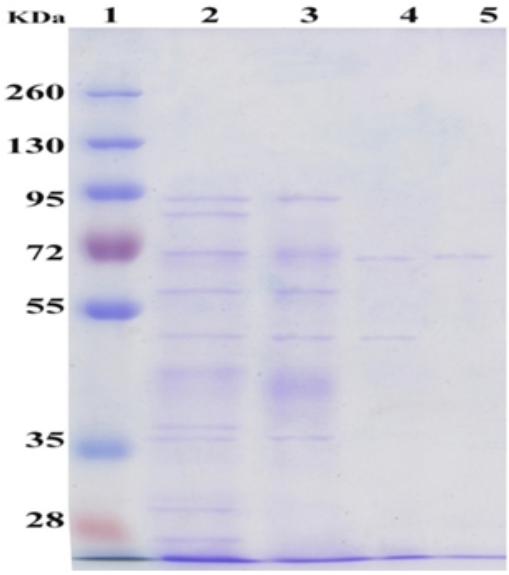

Fig. 4. Electrophoretic analysis of L-glutaminase produced by *Penicillium brevicompactum* NRC 829 at various stages of purification. Separation was performed on a 12 % (w/v) SDS-polacrylamide gel and stained with coomassie brilliant blue. From left to right: lane 1, molecular weight markers; lane 2, Crude-enzyme extracts; lane 3, fractional precipitation by heat treatment for 20 min. at 50ºC, lane 4, Partial purified L-glutaminase on Sephadex G-100; lane 5, purified L- glutaminase on Sephadex G-200

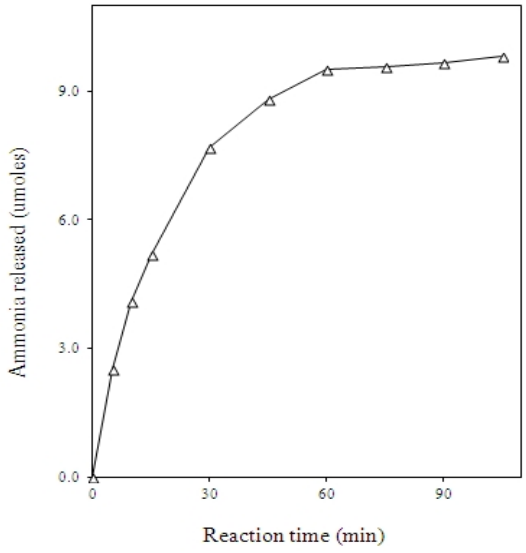

Fig. 5. Activity of purified L-glutaminase as a function of the time of the reaction
Reaction mixture contained: L-glutamine, 10 µmoles; boric-acid borate, 40 µmoles, pH 8.0; extract protein, 22 µg; total vol.0.5ml; temp, 37°C; reaction time, as indicated.

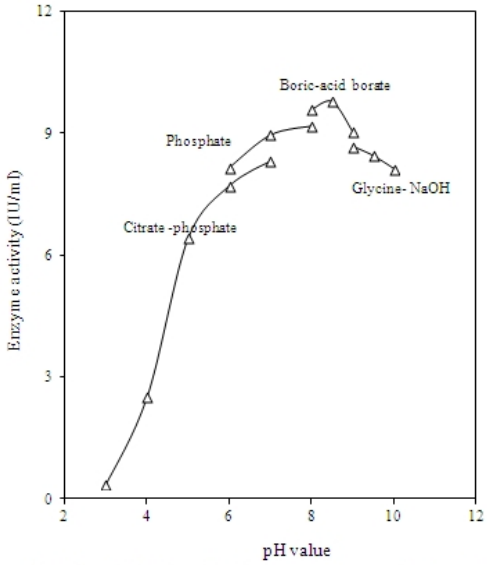

Fig. 6. Activity of purified L-glutaminase as a function of the pH value of the reaction mixture.

Fig. 6. Activity of purified L-glutaminase as a function of the pH value of the reaction mixture

Reaction mixture contained: L-glutamine, 10 µmoles; buffer, 40 µmoles, pH, as indicated; extract protein, 22 µg; temp, 50ºC; total vol. 0.5 ml; reaction time, 30 min.

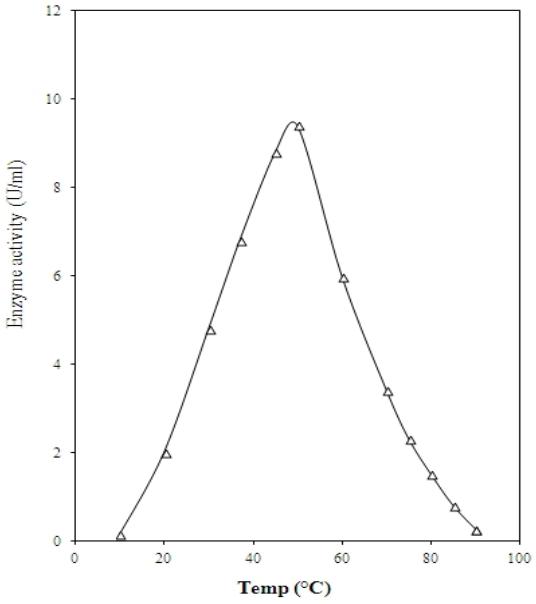

Fig. 7. Temperature dependence of the purified L-glutaminase produced by *P. brevicompactum* NRC 829

Reaction mixture contained: L-glutamine, 10 µmoles; boric-acid borate, 40 µmoles, pH 8.0; extract protein, 22 µg; total vol. 0.5 ml; temp, as indicated; reaction time, 30 min.

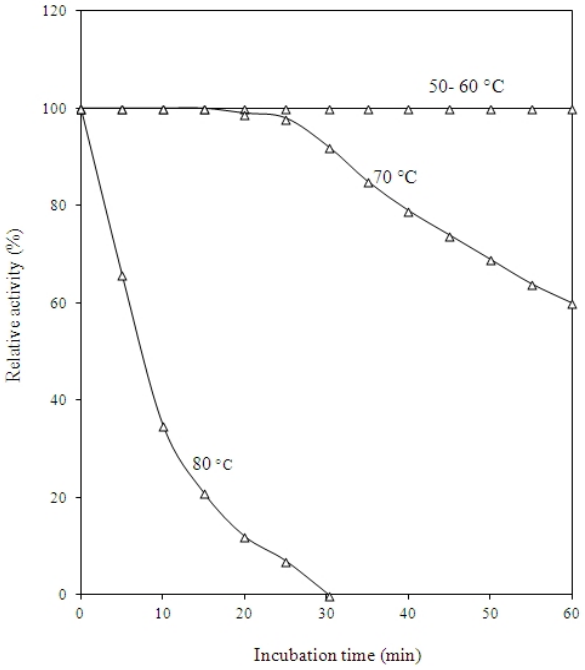

Fig. 8. Heat inactivation kinetics of purified L-glutaminase

Reaction mixture contained: L-glutamine, 10 µmoles; boric-acid borate, 40 µmoles, pH 8.0,; extract protein, 22 µg; temp, 50°C; total vol. 0.5 ml; reaction time, 30 min.

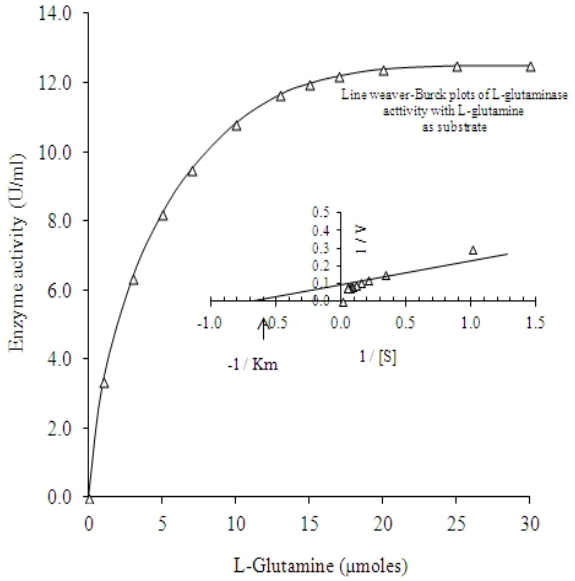

Fig. 9. Effect of L-glutamine concentration on L-glutaminase activity

Reaction mixture contained: µmoles L-glutamine, as indicated; boric-acid borate buffer, 40 µmoles, pH, 8.5; extract protein, 22 µg; total vol. 0.5 ml; temp, 50°C; reaction time, 30 min.

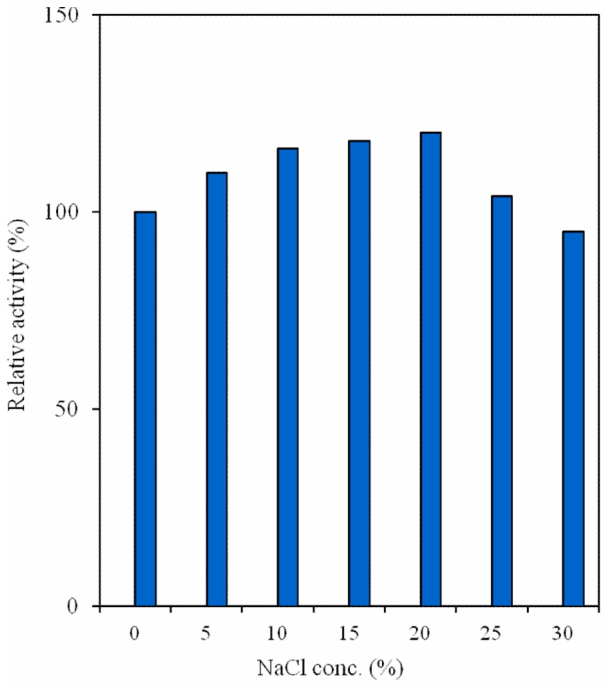

Fig. 10. Effect of different concentrations of NaCl on the purified L-glutaminase
Reaction mixture contained: L-glutamine, 10 µmoles; boric-acid borate buffer, 40 µmoles, pH 8.5; extract protein, 22 µg; temp, 50°C; NaCl, as indicated; total vol. 0.5 ml; reaction time, 30 min.

4. CONCLUSION

For a glutaminase to be ideally suited for use in antineoplastic therapy, it should satisfy a variety of criteria. The selected organism should produce the glutaminase in high yield, and it should be capable of being grown in large quantities on a simple and inexpensive medium. The procedures developed for purification of the enzyme should be as rapid and simplified as possible, providing pure enzyme in high yield. The purified enzyme should have long term stability on storage, maximal activity at a physiological pH, and a K_m for substrate below the concentration of the substrate in the blood. The L-glutaminase we isolated from *Penicillium brevicompactum* NRC 829 meets many of these criteria. The organism grows well on the simple medium described. The enzyme can be purified to homogeneity rapidly and with an overall yield of 48% by a simple procedure involving heat treatment, gel filtration on Sephadex G-100 followed by Sephadex G-200.The pure enzyme has a favorable activity over wide ranges of pH and temperature, high affinity towards L-glutamine, and high thermal stability, which worth further investigations of its proper utilization. In addition, anti-proliferative activity of the enzyme on different cell lines growth, especially the human hepatocellular carcinoma cell line, could be used to develop therapy of different types of tumors.

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COMPETING INTERESTS

Authors have declared no competing interests.

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