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Purification, Characterization and Antitumor Activity of L-asparaginase from *Penicillium brevicompactum* NRC 829

Ali Mohamed Elshafei[1*], Mohamed Mohamed Hassan[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.

Research Article

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ABSTRACT

Aim: The aims of this study were to attempt to extract, purify and characterize of L-asparaginase, an antitumor agent, from *Penicillium brevicompactum* NRC 829.
Study Design: Testing of antitumor activity of L-asparaginase 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 June 2010 and November 2011.
Methodology: *Penicillium brevicompactum* NRC 829, a local isolated strain from Culture Collection of the National Research Centre of Egypt, was grown and maintained on modified Czapek Dox medium. The fresh fungal biomass was thoroughly ground with washed cold sand. The cell contents were extracted with cold 0.1M Tris-HCl pH 8.0, thereafter, the slurry obtained was centrifuged at 5500 rpm for 15 min and the supernatant was directly used as the source of enzyme. The purification of L-asparaginase from crude-enzyme extracts of *P. brevicompactum* was achieved by a sequential multi-steps process starting by heat treatment for 20 min at 50ºC, followed by gel filtration on Sephadex G-100 column, and the most active fractions of L-asparaginase were dialyzed out, lyophilized and then loaded on a Sephadex G-200 column.
Results: An intracellular glutaminase-free-L-asparaginase from *Penicillium brevicompactum* NRC 829 was purified to homogeneity with an apparent molecular mass (M_r) of 94 kDa. The purified enzyme was 151.12 fold with a final specific activity of 574.24 IU/mg protein and about 40% yield recovery. The purified L-asparaginase showed its maximal activity against L-asparagine when incubated at pH 8.0 at 37ºC for 30 min. The

E-mail address: alielshafei@yahoo.com;

enzyme was more stable at alkaline pH than the acidic one and thermally stable up to 60 min at 50-60ºC. L-asparaginase was highly specific for its natural substrate, L-asparagine with a K_m value of 1.05 mM. The activity of L-asparaginase is activated by mono cations and various effectors including $K^{+,}$ Na^{+}, 2-mercaptoethanol (2-ME), and reduced glutathione (r-GSH), whereas it is moderately inhibited by various divalent ions including Hg^{2+}, Cu^{2+}, and Ag^{+}. Results indicated the involvement of sulfhydryl group(s) in the enzyme active site(s). The purified enzyme inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2), with IC_{50} value of 43.3µg/ml.

Conclusion: L-asparaginase purified from *Penicillium brevicompactum* NRC 829 is a potential candidate for medical applications.

Keywords: L-asparaginase; Penicillium brevicompactum NRC 829; purification; kinetic properties; antitumor activity.

1. INTRODUCTION

L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) constitutes one of the most biotechnologically and biomedically important group of therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales (Warangkar and Khobragade, 2010). The enzyme catalyzes the deamidation of L-asparagine to L-aspartate and ammonia (Fig. 1), its antileukemic effect is attributed to the inability of neoplastic blast cells to synthesize L-asparagine from aspartic acid as they lack L-asparagine synthetase. Lymphatic tumor cells need large amounts of asparagine in order to achieve rapid malignant growth. Therefore, the commonest therapeutic practice to treat this condition is to intravenously administer L-asparaginase in order to deplete the blood L-asparagine level and exhaust its supply to selectively affect the neoplastic cells (Theantana et al., 2009; Deokar et al., 2010; Warangkar and Khobragade, 2010). In addition, L-asparaginase plays a central role in the amino acid metabolism and utilization, where, in human body, it acts as a precursor of ornithine in the urea cycle and in transamination reactions forming oxalo acetate in the gluconeogenic pathway leading to glucose formation (Hosamani and Kaliwal, 2011).

L-asparaginase is also being used in food industry to reduce the formation of carcinogenic acryl amides in deep fried potato recipes (Friedman, 2003). Although, L-asparaginase has been found in number of organisms like serum of guinea pig and rodents, chicken liver, yeast, molds, plants and number of bacteria, however, not all of these enzymes are clinically active (Verma et al., 2007). It is well demonstrated that only L-asparaginase obtained from *Escherichia coli* and *Erwinia chrysanthemi* have been used in humans. The therapeutic effect of L-asparaginase from these two bacterial species is accompanied by side effects that might include anaphylaxis, diabetes, leucopoenia pancreatitis, neurological seizures and coagulation abnormalities which may further lead to intracranial thrombosis or haemorrhage. These side effects are partially attributed to the presence of L-glutaminase activity obtained from these sources (Kotzia and Labrou, 2005).

Therefore, it is desirable to search for other L-asparaginase producing microorganisms with novel properties that can produce an enzyme with less adverse effects. Yeast and filamentous fungi are commonly reported in scientific literature to produce L-asparaginase with less adverse effects than prokaryotic microorganisms (Sarquis et al., 2004; Baskar and Renganathan, 2009). In the present study, L-asparaginase was purified and characterized from *Penicillium brevicompactum* NRC 829, the work was then extended to evaluate the antitumor activity of the purified enzyme against different human cell lines.

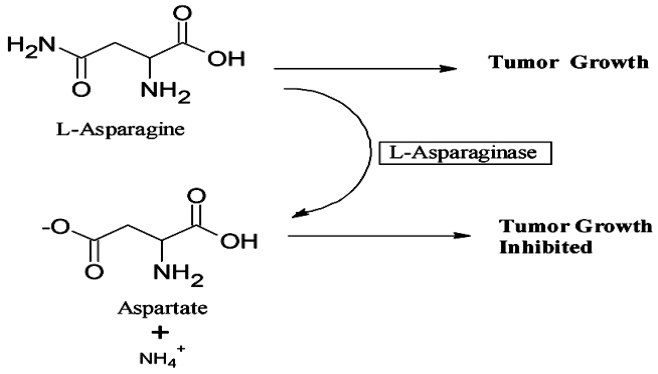

Fig. 1. Schematic illustration of the reaction mechanism of L-asparaginase

2. MATERIALS AND METHODS

2.1 Microorganism

Penicillium brevicompactum NRC 829, a local strain obtained from 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 (Difco, Manual 1972) adjusted to pH 6.0 and supplemented with 2% D-glucose as the only carbon source for growth.

2.2 Chemicals and Buffers

Anhydrous L-asparagine, 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, (1955).

2.3 Growth Conditions for Fungal Culture

The fungal strain was grown on modified glucose Czapek Dox agar slants for seven days at 28ºC. 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 250.0 ml Erlenmeyer flasks, each containing 50.0 ml of sterilized modified glucose 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 (Sebald et al., 1979). The cell contents were extracted with cold 0.1M Tris-HCl 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-asparaginase enzyme assay was performed by a colorimetric method by quantifying ammonia formation in a spectrophotometric analysis using Nessler's Reagent (Gurunathan and Sahadevan, 2011). For routine assay 0.1 ml (35 µg) of diluted enzyme solution was added to 0.4 ml of 0.025 M L-asparagine solution in 0.1 M Tris-HCl 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 diluted with 3.8 ml of distilled water. The, 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 (units per milligram of enzyme) (Roberts, 1976). One unit of L-asparaginase is defined as the amount of enzyme that liberates one micromole (µmol) of ammonia per minute under the standard conditions (pH 8.0, temp 37ºC) (Wriston and Yellin, 1973). The specific activity is defined as the units of L-asparaginase per milligram protein (Bansal et al., 2010).

2.6 Determination of Protein Concentration

Protein content in the crude enzyme preparation was determined according to Bradford, 1976 using bovine serum albumin as the standard. Proteins in the purified fractions were monitored according to Schleif and Wensink, 1981.

2.7 Purification of L-asparaginase

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 cool centrifugation at 5500 rpm (-4ºC) for 10 min (Roberts, 1976).

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 Tris-HCl 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 Tris-HCl 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 Tris-HCl buffer (pH 8.0), at a flow rate of 10 ml/h. The fractions were collected and examined for L-asparaginase 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, 1970. 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 the 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-asparaginase.

2.9 Optimal Reaction Time

This experiment was carried out to identify the optimal incubation time on the L-asparaginase 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-asparaginase 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), Tris-HCl (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 they were incubated under the standard assay conditions. The enzyme kinetics as measured by the Michaelis constant (K_m) is defined as the substrate concentration at half the maximum velocity, the rate of enzymatic reactions, by relating reaction rate to the concentration of a substrate. The Michaelis constant (K_m) value of the purified enzyme was estimated in a range of L-asparagine concentrations of 0.05–30 µmoles. The apparent K_m value of purified L-asparagine was calculated from the Lineweaver-Burk plots relating 1 / V to 1 / [S] (Lineweaver and Burk, 1934).

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^{+}, Ag^{+}, 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) of the salts that were incubated with the purified enzyme for 2 hrs. After the exposure time, enzyme activity in each sample was measured and expressed as a relative activity percentage calculated from the ratio of the specific activity of the treated L-asparagenase to that of the untreated sample.

2.14 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) – Hct 116 (colon cell line) DMEM 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 (Mosmann, 1983).

All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were batch cultured for 10 days, then seeded at concentration of $10x10^{3}$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37ºC for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC 2323, Cornelius, OR, USA). Media were aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of partially purified and purified L-asparaginase dissolved in DMSO to give a final concentration of (100-50-25-12.5-6.25-3.125-1.56 and 0.78 ug/ml). Cells were suspended in RPMI 1640 medium [for Hep-G2- MCF7 - Hct-116 – DMEM-A 549], 1% antibiotic-antimycotic mixture (10,000U/ml potassium penicillin, 10,000µg/ml streptomycin sulfate and 25µg/ml amphotericin B) and 1% L-glutamine in 96-well flat bottom microplate at 37ºC under 5% CO_2. After 48 hrs of incubation, medium was aspirated, 40 l MTT salt (2.5 µg/ml) was added to each well and incubated for further four hours at 37ºC under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 µL of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37ºC. A positive control Adrinamycin (Doxorubicin) [Mw= 579.99] composed of 100 µg/ml was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions (Thabrew et al., 1997).

The absorbance was measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The percentage of change in viability was calculated according to the formula: (Reading of extract / Reading of negative control)$^{-1}$ x 100 A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

3. RESULTS AND DISCUSSION

3.1 Purification of L-asparaginase Enzyme from *Penicillium Brevicopactum* 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-asparaginase on Sephadex G-100 column. The most active fractions (F9-F11) for enzyme activity with specific activity 132.4 IU/ mg, purification fold of about 35 and 63% yield were pooled together, dialyzed against 0.01 M Tris-HCl buffer (pH 8.0), and concentrated by lyophilization (-50ºC).

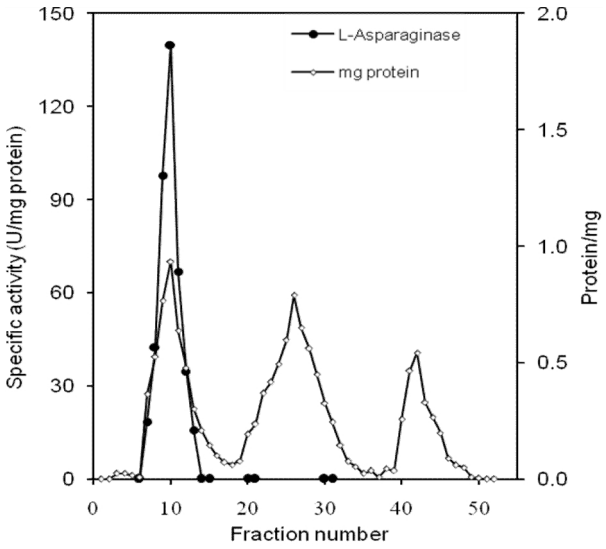

Fig. 2. Elution diagram of L-asparaginase of *P. brevicompactum* from Sephadex G-100

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-asparaginase activity, which fits with only one protein peak, was noticed. The most active fractions (F7-F9) with specific activity 574.24 IU/ mg and about 151-fold purification and 40% enzyme recovery were pooled together, concentrated with lyophilizer and stored at -20ºC.

3.2 Molecular Weight Determination by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE)

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-asparaginase with an apparent molecular weight of 94 kDa (Fig. 4). In this respect, L-asparaginases purified from *Pseudomonas stutzeir* MB-405, *Thermus thermophilus* and *Escherichia coli* were with smaller M_r values ranging from 33-34 kDa, (Manna et al., 1995; Prista and Kyridio, 2001; Soares et al., 2002). Purified L-asparaginase from *Bacillus* sp. (Moorthy et al., 2010), *Streptomyces gulbargensis* (Amena et al., 2010), *Streptomyces albidoflavus* (Narayana et al., 2007) and *S.* PDK2 (Dhevagi and Poorani, 2006) exhibited a molecular weight of 45, 85, 112 and 140 kDa, respectively. Reports on

production and purification of L-asparaginase from *Pseudomonas aeruginosa* by SDS PAGE revealed a peptide chain with molecular weight of 160 kDa (El-Bessoumy et al., 2004).

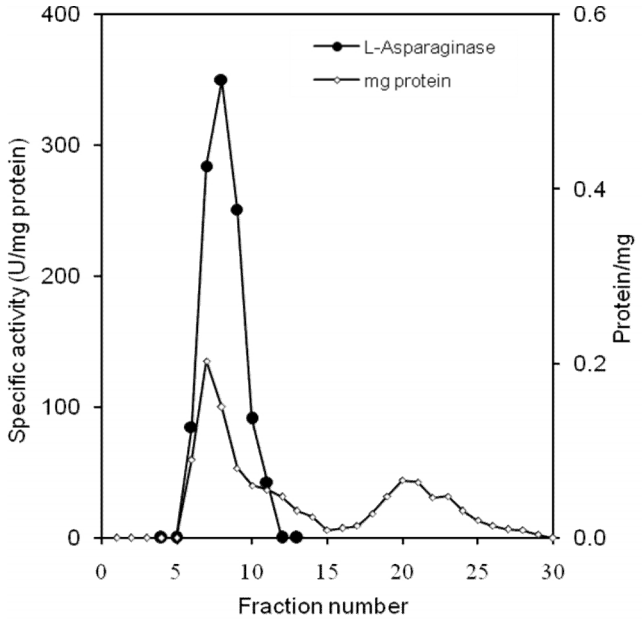

Fig. 3. Elution diagram of L-asparaginase of *P. brevicompactum* from Sephadex G-200

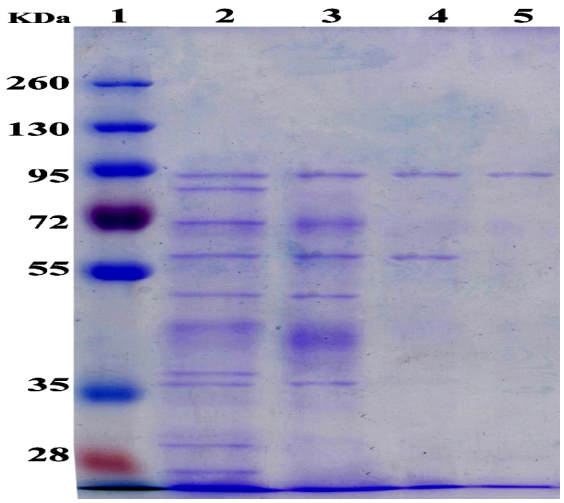

Fig. 4. Electrophoretic analysis of L-asparaginase produced by *Penicillium brevicompactum* NRC 829 at various stages of purification. Separation was performed on a 12 % (w/v) SDS-polacrylamide gel and stained with coomassie brilliant blue. From left to right: lane 1, molecular weight markers; lane 2,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

3.3 Kinetic Properties of the Purified L-asparaginase

3.3.1 Effect of the time

The L-asparaginase activity (Fig. 5), increased as the incubation time increased up to 45 min. After which only a slight increase in enzyme activity was reported. Similar results were recorded for L-asparaginase activity from chicken liver (El-Sayed et al., 2011). In addition, El-Bessoumy et al. (2004) reported that, the incubation of L-asparaginase purified from *Ps. aeruginosa* at 37ºC for different time intervals showed that the activity reached its maximum at 30 min.

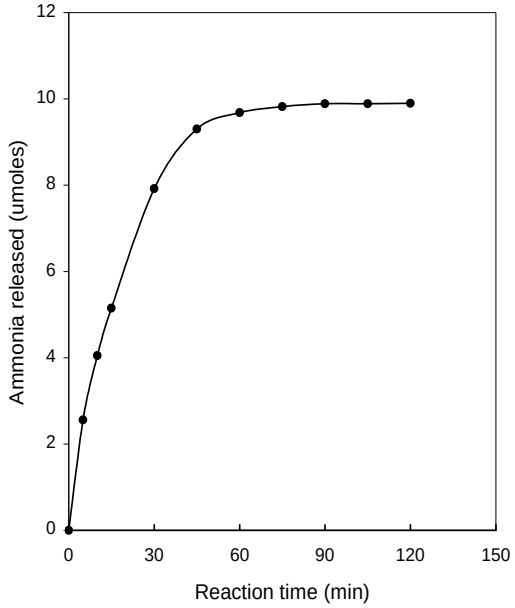

Fig. 5 Activity of purified L-asparaginase as a function of the time of the reaction.

Legend 5: *Reaction mixture contained: L-asparagine, 10 µmoles; Tris-HCl buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; at pH 8.0; 37ºC; reaction time, as indicated.*

3.3.2 Effect of pH on enzyme activity and stability

Results (Fig. 6) revealed that pH 8.0 was the optimal pH for L-asparaginase from *P. brevicompactum* using boric acid-borate buffer. These results coincide with that of Dhevagi and Poorani (2006) who reported the maximal L-asparaginase activity of *Streptomyces sp.* PDK7 was between pH 8.0 and 8.5, and the optimal L-asparaginase activity extracted from *Streptomyces gulbargensis was* 9.0 (Amena et al.,2010). L-asparaginase is one of the amidases that are generally active and stable at neutral and alkaline pH, whereas, pH 5.0 to 9.0 were reported earlier to be optimum for amidase activity (Ohshima et al., 1976). L-asparaginase, purified from marine actinomycete, exhibited maximum activity between pH 7.0 and 8.0 (Basha et al., 2009), membrane bound L-asparaginase from *Tetrahymena pyriformis* acts optimally at pH 9.6 (Triantafillons et al., 1988) and the optimal L-asparaginase activity from *Corynebacterium glutamicum* was reported at pH 7.0 (Mesas et al., (1990). The purified L-asparaginase was more stable in alkaline pH than the acidic one; it retains 100% activity at pH 8.0 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. Moreover, the enzyme retains about 78% of its activity at pH 10. Similar findings were reported for L-asparaginase extracted from *Pseudomonas stutzeri* MB–405 reported to be maximally stable at pH range from 7.5 to 9.5 (Manna et al., 1995). Our results also demonstrated that, L-asparaginase retained about 64 % of its activity after storing at pH 4.0 for 24 hrs. This means that, L-asparaginase of *P. brevicompactum* had higher pH stability over a wide range of pH values.

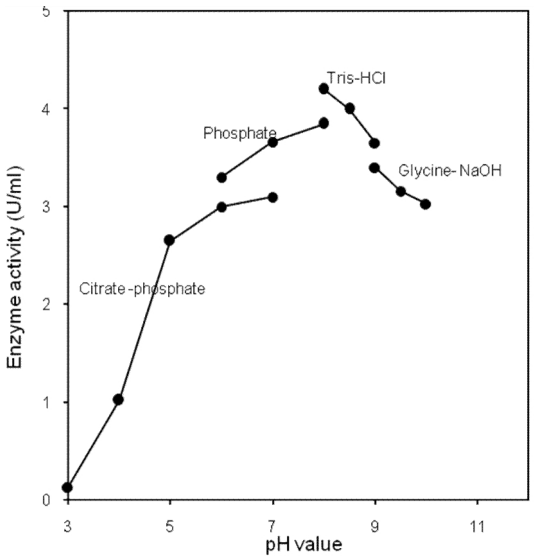

Fig. 6. Activity of purified L-asparaginase as a function of the pH value of the reaction mixture.

Legend 6: *Reaction mixture contained: L-asparagine, 10 µmoles; buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; pH, as indicated; at 37ºC; for 30 min.*

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

Purification step	Total activity(U)	Total protein (mg)	Sp. activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	475 ± 0.51	125 ± 0.43	3.80	100.0	1.0
Heat treatment for 20 min at 50ºC	439.9 ± 0.23	25.12 ± 0.18	17.51	92.61	4.61
Sephadex G-100 L-Asparaginase (F9-F11)	297.9 ± 0.11	2.25 ± 0.13	132.40	62.71	34.84
Sephadex G-200 L-Asparaginase (F7-F9)	189.5 ± 0.18	0.33 ± 0.27	574.24	39.90	151.12

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-asparaginase

Buffer (0.05 M)	pH value	Relative activity (%)
Control	8.0	100
Citrate-phosphate	3.0	38
	4.0	55
	5.0	62
Sodium-phosphate	6.0	75
	7.0	90
Boric-acid borate	8.0	100
	8.5	98
	9.0	96
Glycine-NaOH	9.5	86
	10.0	78

Reaction mixture contained: L-asparagine, 10µmoles; boric-acid borate buffer, 40 µmoles; extract protein, 18 µg; total vol. 0.5 ml; at pH 8.0; 37ºC; for 30 min.

3.3.3 Effect of temperature and thermal stability

The purified L-asparaginase was active at a wide range of temperature from 30ºC to 75ºC with an optimum at 37ºC (Fig. 7), about 35% of L-asparaginase activity was still present at 70ºC, but it lost its activity at 90ºC. Our results were in agreement with a previous study which reported that the optimum temperature for L-asparaginase activity obtained from *Pseudomonas stutzeri* MB-405 was 37º. L-asparaginase from *Erwinia* sp. showed maximum activity at 35ºC (Borkotaky and Bezbaruah, 2002), and maximum activity of L-asparaginase purified from *Streptomyces gulbargensis* was at 40ºC (Amena et al., 2010). Similar results were also reported by Mesas et al. (1990) for L-asparaginase purified from *Corynebacterium glutamicum*. This property of of L-asparagine enzyme makes it most suitable for complete elimination from the body when patient is treated with L-asparaginase *in-vivo*.

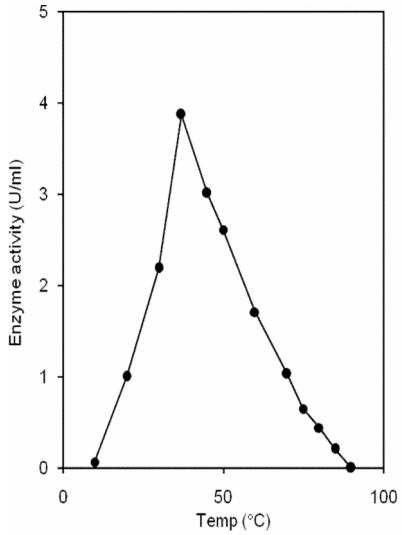

Fig.7. Temperature dependence of purified L-asparaginase produced by *P. brevicompactum* NRC 829

Legend 7: *Reaction mixture contained: L-asparagine, 10 µmoles; Tris-HCl buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; temp, as indicated; at pH 8.0; for 30 min.*

The results of temperature effect on enzyme stability indicated that no significant enzyme activity was lost when it was preincubated at 50ºC to 60ºC for 60 min (Fig. 8). About 30 % of L-asparaginase activity was lost after incubation at 70ºC for 30 min, while a rapid decrease in the enzyme activity (28%) was observed after incubation at 80ºC for 5 min. An earlier study reported no significant loss of L-asparaginase activity purified from *Streptomyces radiopugnans* MS1, when the enzyme was pre-incubated at 40ºC for 60 min (Kumar and Selvam, 2011).

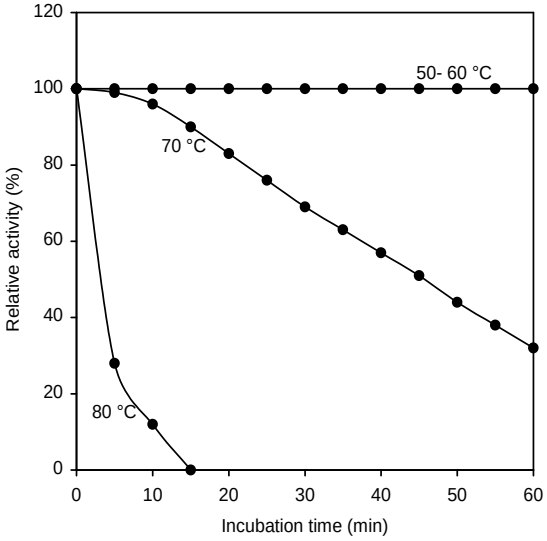

Fig. 8. Heat inactivation kinetics of purified L-asparaginase

Legend 8: *Reaction mixture contained: L-asparagine, 10 µmoles; Tris-HCl buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; at pH 8.0; 37ºC; for 30 min.*

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-asparaginase was found towards its natural substrate L-asparagine while the least activity was obtained with acetamide (Table 3). No activity could be detected against L-glutamine, D-glutamine or NAD. However, L-asparaginase affinity towards acrylamide was quite close to that for L-asparagine. The data indicated that the enzyme extracted from *P. brevicompactum* NRC 829 is very specific to its natural substrate L-asparagine. This property of the enzyme is very essential for the treatment of patients. Our results are in agreement with what has been reported by other studies (Campbel and Mashburn, 1969; Manna et al., 1995).

The K_m of L-asparaginase for L-asparagine was found to be 1.05 mM (Fig. 9). This result indicates the high affinity of L-asparaginase towards its natural substrate, which might relate to its degree of effectiveness against tumors. Higher K_m values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported (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).

Table 3. Substrate specificity of purified L-asparaginase

Substrate (10 µmoles)	Relative activity (%)
L-Asparagine	100
L-Glutamine	0.00
D-Asparagine	0.50
D-Glutamine	00.0
NAD	00.0
Acetamide	03.0
Acrylamide	96.0

Reaction mixture contained: Substrate, 10 µmoles; boric-acid borate buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; at pH 8.0, 37°C; for, 30 min.

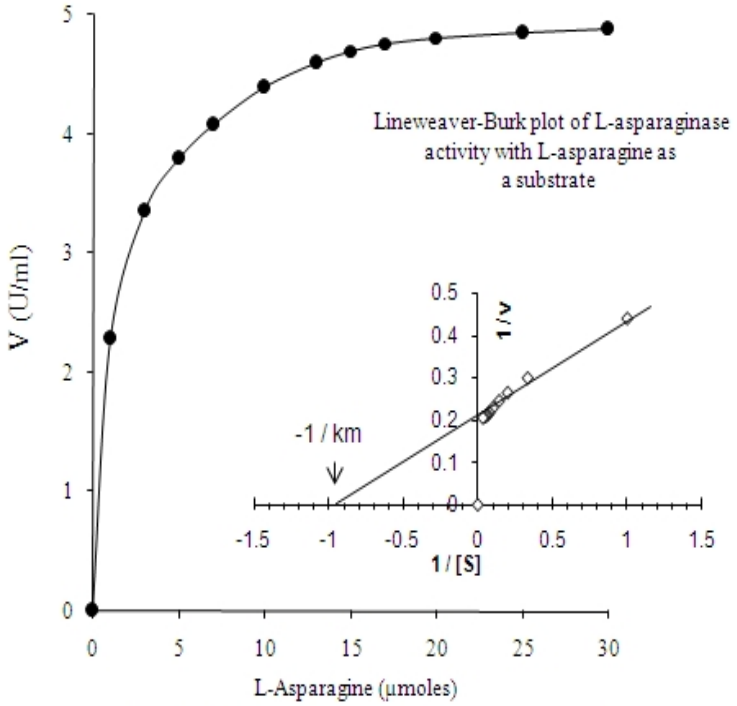

Fig. 9. Effect of L-asparagine concentration on L-asparaginase activity

Legend 9: *Reaction mixture contained: µmoles L-asparagine, as indicated; boric-acid borate buffer, 40 µmoles, extract protein, 18 µg; total vol. 0.5 ml; pH, 8.0; at 37ºCfor 30 min.*

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+}, Cu^{2+} and Ag^{+}. However, the highest inhibition value was recorded with Ag^{+}, which inhibited the enzyme completely at a final concentration of 10^{-2} M, while Na^{+} or K^{+} acting somehow as an enhancer (Table 4). Inhibition of enzyme activity with EDTA possibly suggested that the purified L-asparaginase might be a metaloenzyme. The inhibition of L-asparaginase from marine actinomycete by Cu^{2+} and EDTA was reported in a previous study (Basha et al.,

2009) and L-asparaginase extracted from *Bacillus* sp. was strongly inhibited by EDTA (Mohapatra et al., 1995; Moorthy et al., 2010).

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

Activator or inhibitor	Relative activity (%)		
	10^{-3}M	5 x 10^{-2}M	10^{-2} M
Control	100	100	100
NaCl	107	122	137
KCl	116	130	149
$BaCl_2$	109	100	97
$HgCl_2$	67	48	27
AgCl	34	22	0.0
$CoCl_2$	128	116	109
$CaCl_2$	105	101	96
$CuCl_2$	68	55	28
EDTA	93	85	74
r-Glutathione	104	115	127
2-Mercaptoethanol	101	109	121
Iodoacetate	92	88	75

Reaction mixture contained: L-asparagine, 10 µmoles; boric-acid borate buffer, 40 µmoles, extract protein, 18 µg; compound, as indicated; total vol. 0.5 ml; at pH 8.0; 37°C; for 30 min.

The Inhibition of enzyme activity in the presence of Hg^{2+} might be indicative of essential vicinal sulfhydryl groups (SH-group) of the enzyme for productive catalysis. Furthermore, stimulation of the activation with reducing agents like 2-mercaptoethanol (2-ME), and reduced glutathione (GSH) and inhibition in the presence of thiol group blocking agent namely, iodoacetate provided supplementary confirmation for the role of sulfhydryl groups in the catalytic activity of the enzyme (Table 5). These results are in agreement with results reported for L-asparaginase from *Erwinia carotovora* and *Streptomyces radiopugnans* MS1 (Warangkar and Khobragade, 2010; and Kumar and Selvam, 2011).

Table 5. Anti-tumor activity of crude enzyme extracts of *Penicillium brevicompactum* on the growth of four human tumor cell lines

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

3.3.6 Antitumor activity

Using MTT assay, the in vitro bioassay cytotoxic effect of *Penicillium brevicompactum* NRC 829 enzymes 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-enzymes 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-asparaginase (partialy purified and purified enzyme) lead to a gradual inhibition in the cell

growth as concluded from the low IC_{50} values of 76.4 and 43.3 µg/ml, respectively (Table 6). 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 and reported that AGS and MKN-28 gastric epithelial cells were the most affected.

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

Sample code	IC_{50} (µg/ml)	IC_{90} (µg/ml)	Remarks
Purified L-asparaginase	43.3	82.1	90.2% at 100µg/ml
Partially purified L-asparaginase	76.4	132.2	65.4% at 100µg/ml

4. CONCLUSION

The purified glutaminase-free-L-asparaginase from *Penicillium brevicompactum* NRC 829 has a favorable activity over wide ranges of pH and temperature, high affinity towards L-asparagine, 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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