Purification, characterization and antitumor activity of L-asparaginase from *Penicillium brevicompactum* NRC 829

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**ABSTRACT**

**Aim:** The objective of this work was to focus on the purification and characterization of L-asparaginase, an antitumor agent, from *Penicillium brevicompactum* NRC 829.

**Study design:** Examination of antitumor activity of L-asparaginase against four different 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’s 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 50°C, followed by gel filtration on Sephadex G-100 column. Finally, 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 (Mₐ) of 94 kDa. The enzyme was purified 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 and 37°C for 30 min. The enzyme was more stable at alkaline pH than the acidic one and thermally stable up to 60 min at 50-60°C. L-Asparaginase was found to be highly specific for its natural substrate, L-asparagine with a Kₘ 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 Hg²⁺, Cu²⁺, and Ag⁺. Results obtained indicate 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) in vitro, with IC₅₀ 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* NRC829; 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, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose formation (Hosamani and Kaliwal, 2011).

L-Asparaginase is also being used in food industry in reducing the formation of carcinogenic acrylamides 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, 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 species is accompanied by side effects such as anaphylaxis, diabetes, leucopenia 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 new organisms producing L-asparaginase with novel properties from different sources that can lead to 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 in vitro.

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

2. MATERIAL AND METHODS

2.1 Microorganism

*Penicillium brevicompactum* NRC 829 was 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’s 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 the method of Gomori (1955).
2.3 Growth conditions for fungal culture

The fungal strain was grown on modified glucose Czapek Dox's agar slants for seven days at 28°C. 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 28°C 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, 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 according to the method presented by 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 4°C 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). Incubation was conducted for 30 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1N H₂SO₄. The precipitated protein was removed by centrifugation and 0.2 ml of supernatant was diluted with 3.8 ml of distilled water. Then, 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 (Roberts, 1976). The enzyme activity was expressed as unit (U) /ml. One unit of activity is defined as that amount of enzyme that liberates one micromole (µmol) of ammonia per min under the standard conditions (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 the method of Bradford (1976) using bovine serum albumin as the standard. Proteins in the purified fractions were monitored by the method of Schleif and Wensink (1981).

2.7 Purification of L-asparaginase

2.7.1 Heat treatment

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

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. Then, the most active fractions were pooled, concentrated by lyophilization and stored at -20°C.

2.8 Molecular weight determination by SDS-PAGE
The polyacrylamide separating gel (main gel) (12%) and stacking gel (5%) were prepared according to a protocol proposed by 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, and then destained several times for two hrs in the destaining solution. Finally, the gel was photographed while wet, dried and kept for comparison for calculation of M_r of the purified L-asparaginase.

2.9 Effect of the reaction time

This experiment was carried out to identify the effect of the 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 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 ± 1°C at different pH values in the absence of substrate and then 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 - 90°C. Thermostability studies were carried out by pre-incubating the enzyme at different temperatures (50, 60, 70 and 80°C) for different time intervals.

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 (10µmoles) 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-asparagine concentrations of 0.05–30 µmoles.

2.13 Effect of different metallic salts and various compounds

Different mineral salts of various cations (i.e. Na^+, K^+, Ag^+, Ba^{2+}, Hg^{2+}, Co^{2+}, Ca^{2+} and Cu^{2+}), EDTA (ethylenediaminetetraacetaete), iodoacetate, reduced glutathione and 2-mercaptoethanol were incubated at different concentrations (10^{-3} M, 5 \times 10^{-2} M and 10^{-2} M) with the purified enzyme for 2 hrs. After the exposure time, the relative activity was measured, which expressed as the percentage ratio of the activity of the enzyme incubated with the effector to that of the untreated enzyme.

2.14 Antitumor activity

Cytotoxic effect on different human cell lines (Hep G 2 – MCF 7 – Hct 116- DMEM A-549) 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 37°C for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC 2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample 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
3. RESULTS AND DISCUSSION

3.1 Purification of L-asparaginase enzyme from *Penicillium brevicompactum* NRC 829

The sequential multi-steps purification procedure was summarized in Table 1. This process starts by a partial purification step using heat treatment technique for 20 min at 50°C, followed by gel filtration on Sephadex G-100 column. Finally, the most active fractions obtained from Sephadex G-100 column were dialyzed out, lyophilized and then loaded on Sephadex G-200 column.

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 (-50°C).

The elution profile of the most active fractions collected from Sephadex G-100 and loaded on Sephadex G-200 column was illustrated in Fig. 3, from which 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 -20°C.

3.2 Molecular weight determination by SDS–PAGE

SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) 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 with an apparent molecular weight of 94 kDa that was indicated to the pure preparation of L-asparaginase (Fig. 4). In this respect, L-asparaginas purified from *Pseudomonas stutzeri* MB-405, *Thermus thermophilus* and *Escherichia coli* were with smaller M 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), *S. albidoflavus* (Narayana et al., 2007) and *S. sp.* 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* revealed, by SDS PAGE, a peptide chain with molecular weight of 160 kDa (El-Bessoumy et al., 2004).

3.3 Kinetic properties of the purified L-asparaginase

3.3.1 Effect of the reaction time

The effect of the incubation time on L-asparaginase activity was illustrated in Fig. 5, from which it appears that L-asparaginase activity 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 this concern, El-Bessoumy et al. (2004) reported that, the incubation of L-asparaginase purified from *Ps. aeruginosa* at 37°C for different time intervals showed that the activity reached its maximum at 30 min.

3.3.2 Effect of pH on enzyme activity

Our results (Fig. 6) revealed that the optimal pH for L-asparaginase from *P. brevicompactum* was found to be at pH 8.0 on using boric acid-borate buffer. Thus our 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. These results were also closed...
to the optimal L-asparaginase activity of *Streptomyces gulbargensis* (9.0) reported by Amena et al. (2010). Amidases are generally active and stable at neutral and alkaline pH, whereas, pH 5.0 to 9.0 was reported earlier to be optimum for amidase activity (Ohshima et al., 1976). Basha et al. (2009) reported that L-asparaginase, purified from marine actinomycete, exhibited maximum activity between pH 7.0 and 8.0. Triantafillos et al. (1988) recorded that membrane bound L-asparaginase from *Tetrahymena pyriformis* acts optimally at pH 9.6. Mesas et al. (1990) reported that the optimal L-asparaginase activity from *Corynebacterium glutamicum* was at pH 7.0. This property of the investigated enzyme makes clear that L-asparaginase produced by *P. brevicompactum* NRC 829 has effective carcinostatic property, because the optimum pH for maximal enzyme activity is close to blood pH. Thus, the optimum pH is one of the prerequisites for antitumour activity (Manna et al., 1995).

### 3.3.3 pH stability

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 ± 1°C (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. The enzyme retains about 78 % of its activity at pH 10. Similar findings were reported by Manna et al. (1995) using *Pseudomonas stutzeri* MB–405. The enzyme obtained by *Ps. stutzeri* MB 405 was maximally stable at pH range from 7.5 to 9.5. Our results also demonstrated that, about 64 % of L-asparaginase activity was still obtained 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.

### 3.3.4 Effect of temperature

Fig. 7 shows that the purified L-asparaginase was active at a wide range of temperature from 30°C to 75°C with an optimum at 37°C. At 70°C about 35% of L-asparaginase activity was still present, while at 90°C, it showed maximum loss of enzyme activity. In accordance with our results, Manna et al. (1995) had found 37°C to be the optimum temperature for L-asparaginase activity from *Pseudomonas stutzeri* MB-405. Borkotaky and Bezbaruah (2002) reported that L-asparaginase from *Erwinia* sp. showed maximum activity at 35°C. L-asparaginase purified from *Streptomyces gulbargensis* was found to be maximally active at 40°C (Amena et al., 2010). Similar results were reported by Mesas et al. (1990) for L-asparaginase purified from *Corynebacterium glutamicum*. This property of enzyme makes most suitable for complete elimination of L-asparaginase from the body when tumour patient treated with L-asparaginase *in-vivo*.

### 3.3.5 Thermal stability

The results on the effect of temperature on enzyme stability are presented in Fig. 8. The data indicated that no significant enzyme activity was lost when it is preincubated at 50°C to 60°C for 60 min. At 70°C about 30 % of L-asparaginase activity was lost after 30 min of exposure. While at 80°C, a rapid decrease in the enzyme activity (28%) was observed after only 5 min of exposure. In this concern, Kumar and Selvam, (2011) investigated that, there was no significant loss in L-asparaginase activity purified from *Streptomyces radiopugnans* MS1, when the enzyme was pre-incubated at 40°C for 60 min.

### 3.3.6 Substrate specificity

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. No activity could be detected against L-glutamine, D-glutamine and 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 enzyme makes most essential on the treatment of patients. The observations reported under the present study are in good agreement with the findings of Campbel and Mashburn (1969) and Manna et al. (1995).

### 3.3.7 Determination of K<sub>m</sub>

The apparent K<sub>m</sub> value of purified amidase for L-asparagine was calculated from the Lineweaver-Burk plots relating 1 / V to 1 / [S] (Lineweaver and Burk, 1934). The K<sub>m</sub> 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. The affinity of L-asparaginase to its substrate is related to its degree of effectiveness against tumors. Higher K<sub>m</sub> values 6.6 and 7.0 mM for L-asparaginase from *Lupinus arboreus* and *Lupinus angustifolius*, respectively, has been reported by Chang and Franden (1981). On The other hand, a lower K<sub>m</sub> value (0.058 mM) was obtained for L-asparaginase from *Erwinia chrysanthemi* 3937 (Kotzia and Labrou, 2007).
3.3.8 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 somewhat as an enhancer (Table 4). Inhibition of enzyme activity with EDTA possibly suggested that the purified L-asparaginase might be a metaloenzyme. In agreement with our results, Basha et al. (2009) reported inhibition of L-asparaginase from marine actinomycete by Cu$^{2+}$ and EDTA. In this concern, L-asparaginase extracted from Bacillus sp. was strongly inhibited by EDTA as investigated by Mohapatra et al. (1995) and Moorthy et al. (2010).

Inhibition of enzyme activity in 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 as shown in Table 5. These results are in accordance with that investigated for L-asparaginase from Erwinia carotovora and Streptomyces radiopugnans MS1 by Warangkar and Khobragade (2010) and Kumar and Selvam (2011), respectively.

3.3.9 Antitumor activity

Using MTT assay, the in vitro bioassay cytotoxic effect of Penicillium brevicompactum NRC 829 crude 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] was studied. The IC$_{50}$ values were calculated from SPSS software program.

The results obtained are cited in Table 5, from which it could be noticed that the crude-enzymes extracts have anti-proliferative activity in different cell lines growth in vitro. However, the highest antitumor activity was recorded towards Hep-G 2 (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 (partially purified and purified enzyme) leads to a gradual inhibition in the cell growth as concluded from its low IC$_{50}$ values of 76.4 and 43.3 µg/ml, respectively, as shown in Table 6. Cappelletti et al. (2008) studied in vitro cytocotoxicity of a novel L-asparaginase from the pathogenic strain Helicobacter pylori CCUG 17874 against different cell lines. They reported that AGS and MKN-28 gastric epithelial cells being the most affected.

4. CONCLUSION

The purified glutaminase-free-L-asparaginase from Penicillium brevicompactum NRC 829 has got the favorable activity over wide ranges of pH and temperature, high affinity towards L-asparagine, and high thermal stability which deserve further investigations for its proper utilization. In addition, the results showed that L-asparaginase has anti-proliferative activity in different cell lines growth in vitro, which allows us to conclude that it can be used for new preparations to develop the therapy of different types of tumors.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


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

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

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.

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

Reaction mixture contained: L-asparagine, 10µmoles; boric-acid borate buffer, 40 µmoles, pH, 8.0; extract protein, 18 µg; temp, 37 °C; total vol. 0.5 ml; reaction time, 30 min.
Table 3. Substrate specificity of purified L-asparaginase.

<table>
<thead>
<tr>
<th>Substrate (10 µmoles)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Asparagine</td>
<td>100</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.00</td>
</tr>
<tr>
<td>D-Asparagine</td>
<td>0.50</td>
</tr>
<tr>
<td>D-Glutamine</td>
<td>0.00</td>
</tr>
<tr>
<td>NAD</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetamide</td>
<td>0.03</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>96.0</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Activator or inhibitor</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-3}M</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>NaCl</td>
<td>107</td>
</tr>
<tr>
<td>KCl</td>
<td>116</td>
</tr>
<tr>
<td>BaCl_{2}</td>
<td>109</td>
</tr>
<tr>
<td>HgCl_{2}</td>
<td>67</td>
</tr>
<tr>
<td>AgCl</td>
<td>34</td>
</tr>
<tr>
<td>CoCl_{2}</td>
<td>128</td>
</tr>
<tr>
<td>CaCl_{2}</td>
<td>105</td>
</tr>
<tr>
<td>CuCl_{2}</td>
<td>68</td>
</tr>
<tr>
<td>EDTA</td>
<td>93</td>
</tr>
<tr>
<td>r-Glutathione</td>
<td>104</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>101</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>92</td>
</tr>
</tbody>
</table>

Reaction mixture contained: L-asparagine, 10µmoles; boric-acid borate buffer, 40 µmoles, pH, 8.0; extract protein, 18 µg; temp, 37°C; compound, as indicated; total vol. 0.5 ml; reaction time, 30 min.
Table 5. Anti-tumor activity of crude enzyme extracts of *P. brevicompactum* on the growth of four human tumor cell lines.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hep-G 2</th>
<th>Hct-116</th>
<th>MCF-7</th>
<th>DMEM- A-549</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzymes</td>
<td>65.3%</td>
<td>41%</td>
<td>34.2%</td>
<td>33%</td>
</tr>
</tbody>
</table>

Table 6. Anti-tumor activity of partial purified and purified amidases on the growth of Hep-G2

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>IC_{90} (µg/ml)</th>
<th>IC_{50} (µg/ml)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified L-asparaginase</td>
<td>43.3</td>
<td>82.1</td>
<td>90.2% at 100µg/ml</td>
</tr>
<tr>
<td>Partially purified L-asparaginase</td>
<td>76.4</td>
<td>132.2</td>
<td>65.4% at 100µg/ml</td>
</tr>
</tbody>
</table>
Fig. 2. Elution diagram of L-asparaginase of *P. brevibacterium* from Sephadex G-100

Fig. 3. Elution diagram of L-asparaginase of *P. brevibacterium* from Sephadex G-200
Fig. 4. Electrophoretic analysis of L-asparaginase produced by *P. brevicompactum* NRC 829 at various stages of purification. Separation was performed on a 12 % (w/v) SDS-polyacrylamide 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.

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

Fig. 6. Activity of purified L-asparaginase as a function of the pH value of the reaction mixture.
Fig. 7. Temperature dependence of purified L-asparaginase produced by *P. brevicaespautum NRC 829*

Fig. 8. Heat inactivation kinetics of purified L-asparaginase
Fig. 9. Effect of L-asparagine concentration on L-asparaginase activity
Legend 5: Reaction mixture contained: L-asparagine, 10 µmoles; Tris-HCl buffer, 40 µmoles, pH 8.0; extract protein, 18 µg; total vol. 0.5 ml; temp, 37 °C; reaction time, as indicated.

Legend 6: Reaction mixture contained: L-asparagine, 10 µmoles; buffer, 40 µmoles, pH, as indicated; extract protein, 18 µg; temp, 37 °C; total vol. 0.5 ml; reaction time, 30 min.

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

Legend 8: Reaction mixture contained: L-asparagine, 10 µmoles; Tris-HCl buffer, 40 µmoles, pH 8.0; extract protein, 18 µg; temp, 37 °C; total vol. 0.5 ml; reaction time, 30 min.

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