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ONE STEP PURIFICATION OF GLUTAMINASE FREE L-ASPARAGINASE FROM *ERWINIA CAROTOVORA* WITH ANTICANCEROUS ACTIVTY

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ABSTRACT

L-asparaginase is an enzyme that catalyzes the conversion of L-asparagine to L-aspartate and ammonia. The important application of the L-asparaginase enzyme is in the treatment of acute lymphoblastic leukemia, Hodgkin disease, acute myelocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma. In the present study, the L-asparaginase from *Erwinia carotovora* MTCC 1428 was purified and used for the killing of Hep-2C cell line. Sonication of the resting cells was carried out to release the intracellular L-asparaginase and cell free extract was subjected to acid precipitation. Sulphopropyl Sephadex was used for further purification of enzyme. The 24% yield of the L-asparaginase was obtained after single step purification. The specific activity of the purified enzyme was found to be 0.37 IU/mg and the electrophoresis results suggest that the L-asparaginase of *E. carotovora* MTCC 1428 exists in the form of dimmer of dimmers. Moreover, the purified L-asparaginase from *E. carotovora* MTCC 1428 do not possess any glutaminase activity. The L-asparaginase purified from *E. carotovora* MTCC 1428 showed better *in vitro* toxicity on Hep-2C cell lines (84% survival) in comparison to commercial L-asparaginase preparation (90% survival) obtained from *E. coli*.

Keywords: L-asparaginase, Therapeutic enzyme, Leukemia, Sulphopropyl sephadex chromatography, Cytotoxicity, MTT assay.

INTRODUCTION

The first discovery of the tumor-inhibitory property of L-asparaginase has been done many years ago, with the observation that guinea-pig serum caused prominent regression of the mouse Gardner lymphosarcoma [Kidd 1953]. In the 1960s, Broome reported that L-asparginase activity in guinea-pig serum was responsible for the anti-lymphoma effects [Broome 1961]. Inhibition of incorporation of L-asparagine, which caused alterations in protein and nucleic acid metabolism of the 6C3HED lymphoma cells by the guinea pig containing L-asparaginase, was found to be responsible for the tumor growth inhibition [Sobin and Kidd, 1971]. The final proof that L-asparaginase was the tumor-inhibitory agent of guinea pig serum was furnished by other investigators who isolated the enzyme to homogeneity as judged by immuno-electrophoresis and demonstrated that it was strongly inhibitory to lymphoma tumors [Wriston 1971]. The anti-leukemic effect of L-asparaginase is a result of rapid and complete depletion of the circulating pool of L-asparagine. As in a great number of patients with lymphoblastic leukemia, the malignant cells depend on exogenous source of L-asparagine to be able to survive, mean while, the normal cells are able to synthesize L-asparagine [Narta *et al.,* 2007].

L-asparaginase is widely distributed in nature, not only in animal organs such as liver of guinea pig, placenta, kidney and intestine of beef and horse [Pritsa and Kyridio**,** 2001; El-Sayed *et al.,* 2011], but also in microorganisms such as

Aspergillus tamari, *A. terreus* [Sarquis *et al,* 2004], *E. coli* [Swain *et al.,* 1993; Cornea *et al.,* 2000], *Erwinia aroideae* [Liu and Zajic, 1973], *Pseudomonas stutzeri* [Manna *et al.,* 1995], *P. aeruginosa* [Abdel-Fattah and Olama, 2002], *Thermus thermophius*, *Erwinia crysanthemi* [Kotzia and Labrou, 2005; Kotzia and Labrou, 2006; Michalska and Jaskolski, 2006; Verma *et al.,* 2007; Tabandeh and Aminlari, 2009] and *Staphylococcus* sp. [Prakasham *et al.,* 2007] Among the actinomycetes, several *Streptomyces* species [Narayana *et al.,* 2008], and in plants such as *Glycine max*, *Oryza sativa, Hordenum vulgare* and *Lupinus* sp [Borek and Jaskolski, 2001]. The enzyme's biochemical and kinetic properties were reported to vary with the genetic nature of the microbial strain used [Eden *et al.,* 1990].

In most of the microorganisms, L-asparaginase accumulates as an intracellular (periplasmic, cytoplasmic and membrane bound) product. In L-asparaginase producing strains, the existence of both periplasm and cytoplasm enzyme have been reported [Schwartz *et al.,* 1966]. The study on the localization of any enzyme plays a vital role in the development of a suitable bioprocess [Moorthy *et al.,* 2010].

Most of the asparaginase preparations also exhibit glutaminase activity [Imada *et al.,* 1973] which is responsible for the decrease of glutamine concentration in blood of treated patients. Clinical results have shown that L-asparaginase from *E. coli* causes toxicity and immuno-suppression in addition to development of resistance [Sahu *et al.,* 2007]. This necessitates the studies to find out L-asparaginase from other sources, an enzyme with a low molecular weight, high affinity for substrate and low toxicity. The present paper is devoted to the purification of glutaminase-free L-asparaginase from *E. carotovora* MTCC 1428 and comparative evaluation of the antileukemic effect on the cancer cell line with commercial preparation.

MATERIALS AND METHODS

1. Chemicals

All chemicals were procured from Himedia, Merck and SDfine, India and were of analytical grade.

2. Microorganism and maintenance of culture

The culture of *E. caratovora* MTCC 1428 was procured from Department of Biotechnology, Himachal Pradesh University, Shimla-5. The culture of *E. caratovora* MTCC 1428 was maintained on medium containing (%, w/v) casein enzyme hydrolysate (tryptone) 1.5, peptone 0.5, NaCl 0.5 and agar 2.0 (pH 7.0) slants containing 1.0 % (w/v) L-asparagine or in 20% (v/v) glycerol stocks and sub culturing was done periodically.

3. Production of L-asparaginase

The *E. caratovora* MTCC 1428 was cultivated on soluble components of medium in 250 mL Erlenmeyer shake flasks under submerged conditions. The preculture was prepared in a medium (pH 7.0) containing (%, w/v) tryptone 1.0, peptone 1.0 and L-asparagine 0.1 by inoculating the medium with loopful of culture and incubated it in a temperature controlled orbital shaker (25^{o}C, 150 rpm). 12 h old, 4% (v/v) preculture was used to inoculate the production medium (L-asparagine 0.6 %). The culture was allowed to grow for 14 h under controlled conditions. The cells were harvested by centrifugation (10,000g for 10 min, 4^{o}C) and cell pellet was washed with potassium phosphate buffer (pH 8.5, 0.05 M).

4. L-asparaginase assay

The L-asparaginase from *E. caratovora* MTCC 1428 was found to be intracellular in nature and hence the resting cells suspended in potassium phosphate buffer (0.05 M, pH 8.5) were used for the enzyme assay. Potassium phosphate buffer 1.45 mL (0.05 M, pH 8.5), cell suspension (50 µL) of known dcw and 500 µL of 10 mM substrate (L-asparagine) prepared in potassium phosphate buffer (0.05 M, pH 7.5) were incubated at 37°C for 15 min. The reaction was stopped by the addition of 500 µL chilled TCA (15% w/v). A set of control was also run and from the reaction mixture, 1 mL was withdrawn and amount of released ammonia was measured by ammonia hypochloride method [Fawcett and Scott, 1960].

5. Protein estimation

The protein contents of the sample were determined by the method described by Bradford

[1976] using bovine serum albumin as a standard.

6. Definition of enzyme activity

L-asparaginase in intact cells: The L-asparaginase unit (U) has been defined as the μ moles of ammonia released/ mg of dcw/ min under standard assay conditions.

L-asparaginase in cell free extract: The L-asparaginase unit (IU) has been defined as the μ moles of ammonia released/ mL/ min under standard assay conditions.

Specific activity of L-asparaginase: IU/mg of proteins.

7. Purification of L-asparaginase of E. carotovora MTCC 1428

All the experiments pertaining to purification of L-asparaginase were carried out at 4°C using potassium phosphate buffer (0.05 M and pH 5.5, 6.3 and 7.0). Sulphopropyl Sephadex chromatography was performed and protein eluted during chromatography was monitored by measuring A_{280} nm using UV-VIS spectrophotometer. The L-asparaginase activity and protein content in each fractions obtained were determined which were further used for the estimation of specific activity, yield and recovery. The SDS-PAGE [Laemmli 1970] analysis of the fractions obtained from the suulphopropyl sephadex chromatography was done to assess the purity of the enzyme. Native–PAGE [Bollag and Edelstein, 1991] was also performed to determine the molecular mass of the L-asparaginase from *E. carotovora* MTCC 1428.

8. Preparation of cell free extract

The resting cell suspension (60 ml, 50 mg/mL) was supplemented with 1 mM each of DTT and EDTA and subjected to 5 cycles (1 min on and 1 min off) the content was withdrawn and centrifuged to remove cell debris at 15,000g at 4°C for 30 min. The amount of protein released and L-asparaginase activity in the cell free extract was determined and was used for further purification.

9. Sulphopropyl sephadex chromatography

Sulphopropyl (SP) Sephadex is a strong cationic exchanger and was earlier used for the purification of L-asparaginase [Krasotkina *et al.,* 2004]. The column was equilibrated with phosphate buffer (0.1 M, pH 5.5). The pH of the 50 mL of cell free extract was adjusted to 5.5 by using 0.1 N HCl and the suspension was left for 45 min at 4°C with continuous stirring for partial precipitation on contaminating proteins. The content was centrifuged (15,000g, 30 min) at 4°C and the soluble fraction of the 50 mL cell free extract was loaded on to a SP sephadex column. The column was consecutively washed with phosphate buffer of pH 5.5 and pH 6.3, until the effluent was found to be devoid of any protein. The L-asparaginase protein was eluted with the phosphate buffer (0.1 M, pH 7.0) and fractions of 1.0 mL were collected. The protein and enzyme activity of each fraction were determined. The purity of L-asparaginase was assessed by SDS and native-PAGE under standard conditions.

10. Analysis of the gel

The images of gel were recorded in a gel documentation system (Alpha Innotech Corporation, USA). The molecular weight analysis was done using Alpha Digi Doc RT and Alpha Ease FC software.

11. Substrate specificity of purified L-asparaginase from E. carotovora MTCC 1428

Different substrates were used to investigate the specificity of L-asparaginase form *E. carotovora* MTCC 1428 towards these substrates (L-asparagine, D-asparagine, DL-asparagine, and L-glutamine). The substrates were prepared in potassium phosphate buffer of pH 8.5 (0.25 M) at 40 mM concentration. The reaction mixture was incubated for 15 min and L-asparaginase activity in each case was determined.

12. In vitro cytotoxicity assay of L-asparaginase from E. carotovora MTCC 1428

The purified L-asparaginase from *E. carotovora* MTCC 1428 was lyophilized and used for the cytotoxicity assay. The commercial preparation of *E. coli* was procured from Sigma Aldrich Inc USA. The *in vitro* cytotoxicity assay of the L-asparaginase from *E. carotovora* MTCC 1428 was performed on mammalian transformed cell line Hep-2C that is a derivate of human cervix carcinoma HeLa cells. The cells were trypsinized from a confluent monolayer culture obtained in a 25 cm^2 canted neck flask. The confluent monolayer of the cells was washed twice with PBS, pH 7.2 followed by exposure to Trypsin-

EDTA (100 mg% EDTA and 125 mg% Trypsin 1: 250; Sigma Chemical Co., St. Louis, USA) disaggregating solution for 2 min. The disaggregating solution was completely removed by decantation and the enzyme solution-treated flask was incubated at 37°C for 3 min. The disaggregated cells were re-suspended in appropriate volume of DMEM supplemented with FCS (10%, v/v) and adjusted to a cell density of 4 X 10^3 cells/mL. Uniform volume of Hep-2C cell suspension (200 µL/well) was poured in the selected wells (60 wells) of a 96-wells tissue culture plate. The columns were marked, and in wells under each of the columns the filter sterilized L-asparaginase prepared in potassium phosphate buffer (0.25M, pH 8.5) was dispensed to achieve final concentration of 0.05, 0.15 and 0.25 IU. The cells treated with the L-asparaginase were incubated in CO_2 incubator with 95% humidity at 37°C for 24 h. Each of the L-asparaginase concentration was tested in quadruplicates and mean values were calculated after MTT assay (using 5 mg/mL in potassium phosphate buffer, 0.25 M pH 8.5) of MTT compound. Appropriate controls with no L-asparaginase but containing appropriate amount of potassium phosphate buffer (used to prepare stock of L-asparaginase) and heat denatured enzyme as negative control were also included to see their effect on the viability of the proliferating cells cultured *in vitro*.

RESULT AND DISCUSSION

1. Relaese of L-asparaginase from resting cells and purification of L-asparaginase from E. carotovora MTCC 1428

The resting cells of *E. carotovora* MTCC 1428 were subjected to sonication for 5 cycles. The released L-asparaginase and protein was found to be 1.41 IU and 17.80 mg/mL, respectively in the cell free extract of *E. carotovora* MTCC 1428. The biggest loss of enzyme occurred at initial step of cell free extract preparation. The recovery of L-asparaginase of *E. carotovora* MTCC 1428 was found to be 70% after sonication. The recovery of enzyme was reported to be more than 80% by 10 min sonication in case of recombinant strain of *E. coli*. [Krasotkina *et al.,* 2004]. Crude L-asparaginase extract of *Thermoactinomyces vulgaris* 13 MES were prepared by grinding the cells with sand, alumina or glass beads, by rapid freezing and thawing, by rapid mixing and also by exposure to ultrasonic waves [Mostafa and Ali, 1983].

2. Acid precipitation

The cell free extract obtained after sonication was centrifuged at 15,000 rpm at 4ºC for 30 min. The pellet was discarded and 50 mL supernatant (1.41 IU, 17.80 mg/mL protein) was taken for acid precipitation. The pH of the supernatant was adjusted to 5.5 with 0.1 N HCl and kept for continuous stirring at 4ºC for 45 min. The contents were centrifuged at 15,000 rpm at 4ºC for 30 min to remove the precipitated contaminating proteins and supernatant was used to determine the enzyme activity and protein content. The L-asparaginase activity was found to be 0.825 IU with 6.25 mg/mL protein after acid precipitation. The acid precipitation was also done for the one step purification of recombinant L-asparaginase from *E. coli* by Krasotkina *et al.,* [2004].

3. Sulphopropyl sephadex chromatography for purification of L-asparaginase by E. carotovora MTCC 1428

The protein elution profile in various fractions of SP Sephadex chromatography has been shown in Fig.1. The ion exchange chromatography resulted in 24% yield and 4.7% fold purification (Table I.). These results suggest that the L-asparaginase polypeptide contained large proportions of positively charged amino acids and thus it bound to negatively charged matrix. The possible reason for single step purification might be the pI value of wild L-asparginase which has reported to be in a range of 8.5-8.7 [Moola *et al.,* 1994]. Hence, at acidic pH, this enzyme retains positive charge and can be exchanged easily by a strong cation exchanger. However, the most of the contaminating proteins with acidic pI value remain unbound and gets eluted from the column.

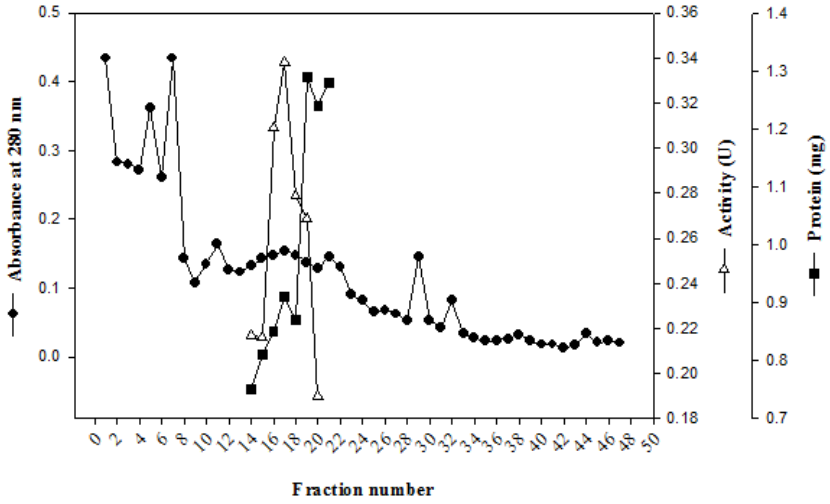

Figure. 1 ***Elution profile of sulphopropyl sephadex chromatography***

Table I. ***Summary of purification of L-asparaginase from E. carotovora MTCC 1428***

Stage	Activity (IU)	Protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Cell free extract	1.41	17.80	0.08	1.0	100
Acid precipitation (5.5 pH)	0.823	6.25	0.13	1.62	59
Purified enzyme	0.34	0.91	0.37	4.70	24

A single chromatographic step, result in the 4.7 fold increase in the specific activity of the enzyme compared to cell free extract. The purification fold of L-asparaginase from a recombinant strain of *E. coli* has reported to be 21 [Krasotkina *et al.,* 2004]. The partially purified L-asparaginase from Coliform bacteria had the purification fold of 2.15 with 20% yield after 20-40% ammonium sulphate precipitation [Shah *et al.,* 2010]. The purification of the enzyme from *P. aeruginosa* by sephadex G-100 gel filtration and SDS-PAGE analysis of the protein was performed by El-Bessoumy *et al.,* [2004]. Dhevagi and Poorani, [2006] purified the L-asparaginase from marine actinomycetes and with purification fold (82.98%) and yield (2.18%) after the simultaneous purification steps of ammonium sulphate precipitation (45-85%), sephadex G 50 filtration and sephadex G 100 filtration.

4. Establishment of the purity of L-asparaginase by polyacrylamide gel electrophoresis

In order to check the purity of the enzyme, SDS-PAGE was conducted [Laemmli 1970]. The analysis of the gel, for the active fraction of SP sephadex column chromatography, revealed two distinct bands of molecular weights 40.2 and 39.8 kD (Fig. 2a). However, the analysis of the native-PAGE for the fraction of SP column chromatography indicated one band of the molecular weight of 160 kD (Fig 2b). These results suggest that L-asparaginase from *E. carotovora* MTCC 1428 was in the form of dimer of dimers and this enzyme seems to be functional in the tetrameric configuration. Mezentsev *et al.,* [2007] and Cammack *et al.,* [1972], analyzed the subunit interface in dimers and tetramers of *E. carotovora* L-asparaginase and confirmed general notion of the tetramer structure of L-asparaginase as a dimer of dimers.

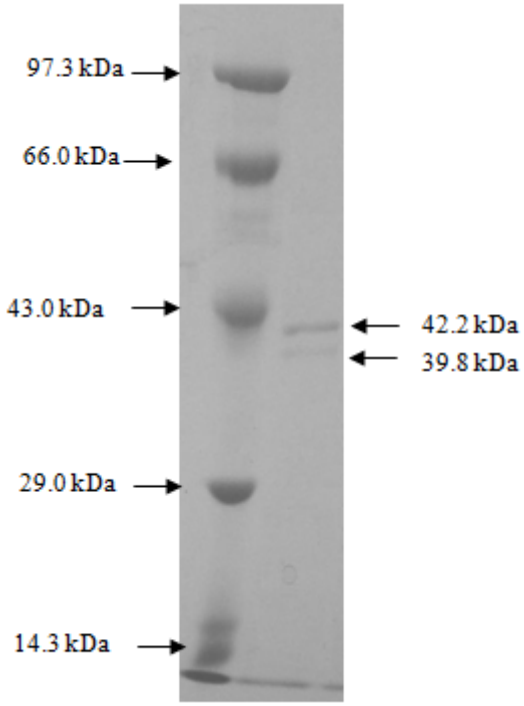

Figure. 2a ***SDS-PAGE of purified L-asparaginase from E. carotovora MTCC 1428***

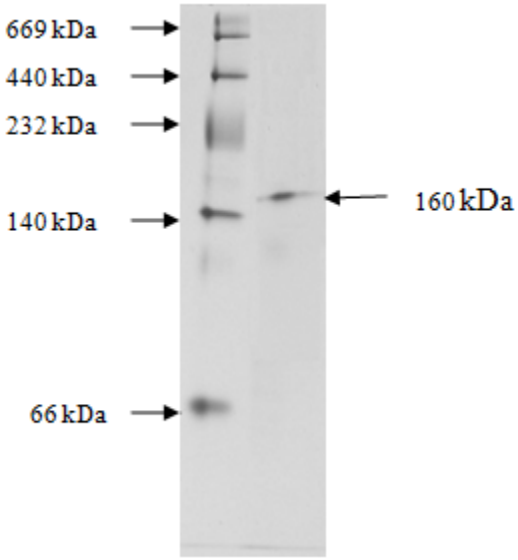

Figure. 2b ***Native-PAGE of purified L-asparaginase from E. carotovora MTCC 1428***

5. Substrate specificity

The activity of enzyme was determined with different substrate (L-asparagine, L-glutamine, D-asparagine and DL-asparagine) at 10 mM concentration to find out the most specific substrate for the purified L-asparaginase of *E. carotovora* MTCC 1428. It was found that the L-asparagine was most suitable substrate for the L-asparaginase of *E. carotovora* MTCC 1428. The purified enzyme exhibited 0.645 IU of L-asparaginase activity (Fig. 3). Moreover, it does not possess glutaminase activity. The substrate specificity for L-asparaginase by *E. carotovora* was determined by Howard and Carpenter, (1972) and they found that the relative specificity of L-asparaginase towards the L-asparagine was maximum among all the substrates used.

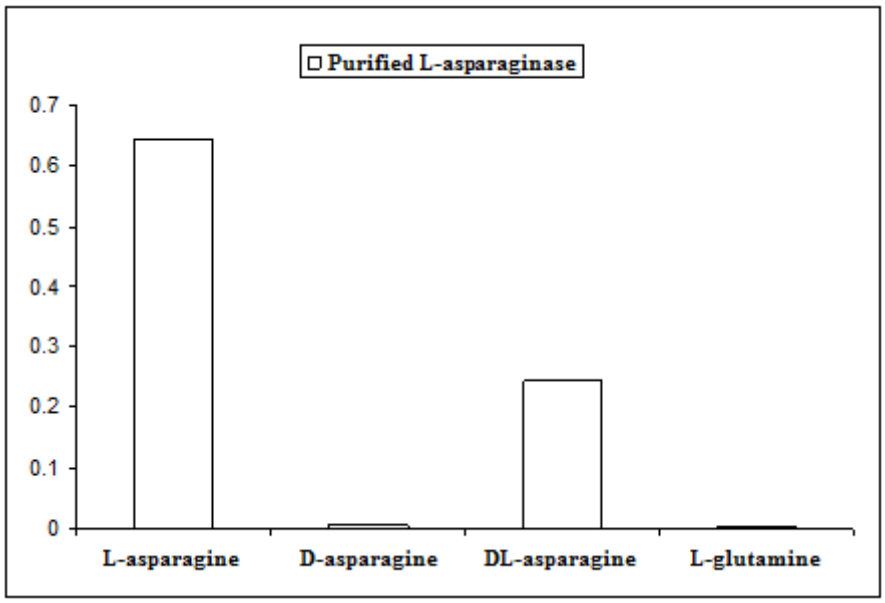

Figure. 3 ***Substrate specificity of purified L-asparaginase from E. carotovora MTCC 1428***

6. In vitro cytotoxicity assay of L-asparaginase from E. carotovora MTCC 1428

The purified L-asparaginase enzyme from *E. carotovora* MTCC 1428 and the commercial preparation (*E. coli*) have been subjected to cytotoxic activity *in vitro* on the Hep-2C cell line. The cytotoxic activity of the purified L-asparaginase from *E. carotovora* MTCC 1428 and commercial preparation from *E. coli* were found to be similar at higher concentrations (Fig. 4). However, the L-asparaginase purified from *E. carotovora* MTCC 1428 showed better toxicity on Hep-2C cell line (84% survival) in comparison to commercial L-asparaginase preparation (90% survival) obtained from *E. coli*. The sensitivity of the cell line to both L-asparaginases (purified and commercial preparation) appeared to be dose dependent, resulting in the significant decrease in viable cells. The treatment of cancerous cell line with increasing concentration of L-asparaginase (upto 0.25 IU) resulted in appreciable inhibition of cell growth.

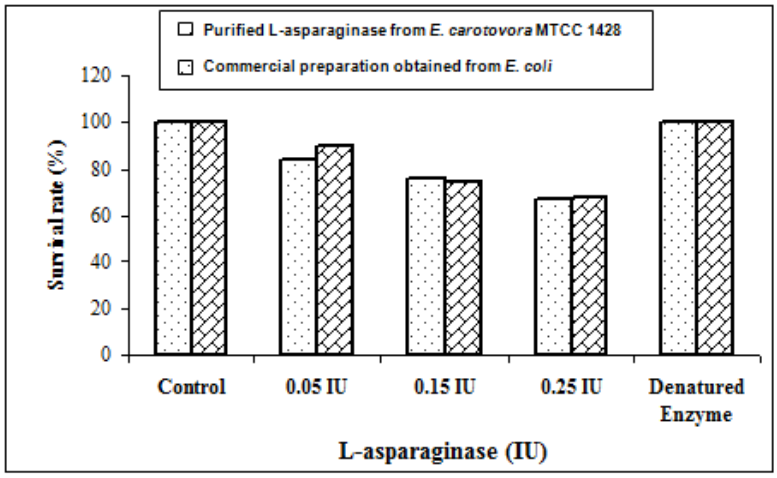

Figure. 4 ***Cytotoxicity of purified L-asparaginase from E. carotovora MTCC 1428 and commercial preparation obtained from E. coli towards Hep-2C cells***

Cell cycle arrest and apoptosis of leukaemia cells induced by L-asparaginase from *E. coli* was observed by Ueno *et al.,* [1997]. Deprivation of L-asparagine from the culture of L5178Y cells by L-asparaginase caused the fragmentation of chromosomal DNA of the leukemia cells within 24 h. Prior to the degradation of DNA, cell cycle of L5178Y cells were found to be arrested in G1 phase, and evidence of the DNA strand breaks was initially observed in G1 phase cells as early as 8 h after the asparaginase treatment. Therefore, apoptosis of leukemia cells induced by L-asparaginase was found to be an event that has been associated with the cell cycle arrest in G1 phase. Ando *et al.,* [2005] have reported the selective apoptosis of natural killer cells tumors by L-asparaginase. Cappelletti *et al.,* [2008] studied *in vitro* cytotoxicity of a novel L-asparaginase from the pathogenic strain *H. pylori* CCUG 17874 against different cell lines. They reported that AGS and MKN 28 gastric epithelial cells being the most affected. The antitumor activity of L-asparaginase from chicken liver was investigated by EI-Sayed *et al.,* [2011]. The enzyme inhibited the growth of the two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (Het-116) with IC_{50} value of 8.38 µg/mL and 4.67 µg/mL, respectively. However, IC_{50} was greater than 10 µg/mL/well for MCF7 (breast carcinoma) cell line. L-asparaginase was identified as the factor responsible for the cell-cycle inhibition of fibroblasts and gastric cell line [Scotti *et al.,* 2010]. Its effect on cell-cycle was confirmed by inhibitors, a knockout strain and the action of recombinant L-asparaginase on cell lines. Interference with cell-cycle *in vitro* depended on cell genotype and was related to the expression levels of the concurrent enzyme asparagine synthatase. L-asparaginase from pathogenic bacteria *E. coli* showed cytotoxicity to U937 cell line with IC_{50} 0.5±0.19 IU/ml, and selectivity index (SI=7.6) about 8 time higher selectivity over the lymphocyte cells [Aljewari *et al.,* 2010].

CONCLUSION

The L-asparaginase has widely been used for the treatment of acute lymphoblastic leukemia. However, the presence of glutaminase activity in L-asaparaginase preparation results in adverse reaction in body. In the present study, an attempt has been made to purify L-asparaginase from *E. carotovora* MTCC 1428 in single step and its effect on cancerous Hep-2C cell line also has been assessed. This L-asparaginase could be used against for the treatment of various malignant diseases.

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