PARTIAL PURIFICATION AND CYTOTOXIC ACTIVITY OF L-ASPARAGINASE ISOLATED FROM *Escherichia coli*

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Abstract

L-Asparaginase (E.C.3.5.1.1) is an important natural product that possesses a broad spectrum of antitumor activity. In the present study, L-asparaginase partially purified from local isolate of *Escherichia coli* that were grown aerobically for four hours and anaerobically for 18hrs on M9 medium contain L-asparagine as sole nitrogen source. Extraction of the enzyme was done by sonication. Qualitative and quantitative assays for *L*-Asparaginase production were determined using colorimetric and nesslerization methods respectively. The purification steps involve dialysis of crude extract and DEAE-Cellulose Ion exchange chromatography. The enzyme was purified 3.39-folds and showed a final specific activity of 0.18 U/mg with a 41% yield. The crude extract and DEAE-Cellulose fractions showed slight growth inhibition against RD cell line (human rhambdomyo sarcoma).

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(E.C.3.5.1.1)

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M9

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.DEAE

.41% . U/mg 3.9

)

.RD cell line (

Introduction

Deamidation of L-asparagine by extracts of *E. coli* was first reported in 1957 [١]. Later, Mashburn and Wriston observed that L-asparaginase (L-asparag-ine amidoh-ydrolase, Enzyme Commission 3. 5. 1. 1) purified from cell extract of *E. coli* has an antitumor activity similar to that of guinea pig serum [2]. L-asparaginase belongs to an amidase group that hydrolyses the amide bond in L-asparagine to aspartic acid and ammonia. Unlike the normal cells, neoplastic cells cannot synthesize L-asparagine due the absence of L-asparagine synthetase. Therefore, they obtain the required asparagine from circulating pools. For this

reason, intravenous injection of free enzyme results in selective neoplastic cell death, directly by the depletion of circula-ting asparagine levels or indirectly from some other metabolite of the asparaginase reaction [3, 4, 5].

L- asparaginase has been successfully applied to the treatment of several diseases such as lymphocyte sarcoma and leukemia [6, 7, 8]. Clinical studies have demonstrated higher recovery efficiency for leukemia patients treated with *L*-asparaginase than those with IL-2 [9]. *L*-asparaginase is an important anti-cancer drug and was the focus of intensive investigations during 1970s that made large-scale industrial manufacture of this enzyme practical in both Japan and the United States from wild type *E. coli* strains [10]. The precise mechanism of its action is still unknown although hydro-lysis proceeds in two steps via a beta-acyl-enzyme intermediate (Figure 1) [11].

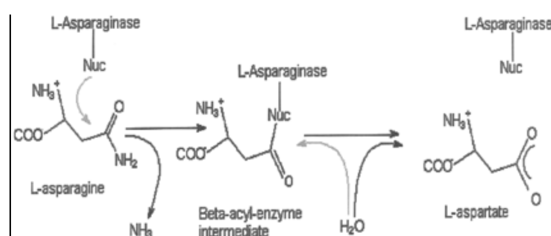

Figure 1: Schematic illustration of the reaction mechanism of L-asparaginase. [11]

The aim of this study is isolation and purification of L-Asparaginase from local isolate of *E.coli* and determination of its antitumor activity against RD cell line (human rhabdomyo sarcoma) *in vitro*.

Materials and methods

Microorganism media and culture conditions

The bacterial strain of *E.coli* kindly provided by the central laboratory, ministry of health. *E. coli* was grown at 37°C in shaker incubator for 4h then transferred to anaerobic jar for 18h at 37°C in M-9 medium containing (per 1L distilled water): Na_2HPO_4, 6.0g; KH_2PO_4, 3.0g; NaCl, 0.5g; L-asparagine, 5.0g; 1mol/l of $MgSO_4.7H_2O$, 2.0 ml; 0.1mol/l; $CaCl_2.2H2O$, 1.0ml; 20% Glucose stock, 10.0ml; pH(7), [12]. At the end of incubation time, culture was centrifuged 3000rpm/15min at 5°C, and then pelleted cells washed twice with sterile distilled water. Sonication for washed cells was done (30sec sonication, 1min stop, for 10 cycles) in a sonicator (Sanyo). After sonication, centrifugation was done 3000 rpm/15min at 5°C. The supernatant was stored at 4°C in order to be used for enzyme assay and purification [13].

Enzyme assay

Qualitative enzyme assay was achieved by cultivation of *E. coli* on modified M-9 medium agar plate, which contains 2g/l phenol red at 37°C overnight then pink zone around the bacterial growth was observed, which indicate the presence of L-asparaginase [14].

Quantitative enzyme assay achieved by direct nesslerization method [15]. In brief, 0.1ml of enzyme sample was mixed with 2.5 ml of 0.1 mM asparagine (pH8.6), incubated at 37°C for 30min, the reaction terminated by addition of 0.5ml of 1.5N tricholoacetic acid ,then centrifuged 5000rpm for 15min. One hundred microliter of supernatant was mixed with 4.4ml of distilled water and 0.5ml of nessler's reagent [16], vortex then measured by spectrophotometer at 436nm. In blank, 2.5ml of 0.1M Tris-Hcl (pH8.6) used instead of L-asparagines. One L-asparaginase unit (IU) is defined as the enzyme amount, which liberate 1μmol of the ammonia/min under optimum conditions. The protein concentration was determined by lowery method (1951) [26] using bovine serum albumin as a standard, (Figure 2). Standard curve for ammonia liberation was bone by measuring the absorbance of gradual concentrations of ammonium sulphate at 436nm, (Figure 3). The enzyme activity (U/ml) calculated by the equation: concentration of liberated ammonia (μg/ml)/reaction time (min) × enzyme sample volume (ml) [17].

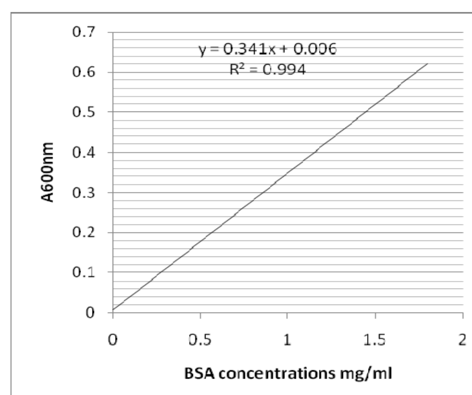

Figure 2: Bovine serum albumin standard curve.

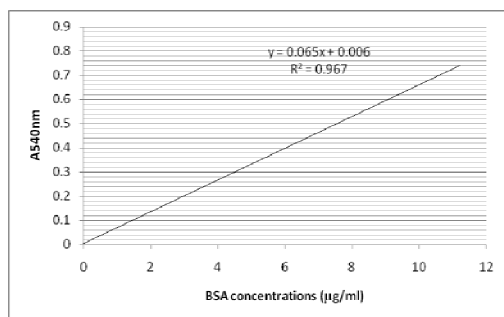

Figure 3: Standard curve for ammonia liberation was bone by measuring gradual concentrations of ammonium sulphate at 436nm

Purification of asparaginase

The supernatant stored following sonication was dialysed against 0.1M of Tris buffer (pH8.0) with three times buffer changes. Ion exchange chromatography was done for dialysed sample using DEAE-cellulose column (9×3cm) equilib-rated and washed with 150ml of 0.1M Tris-Hcl pH8.0 and eluted with stepwise 0-0.5M of ammonium sulphate in 0.1M Tris-Hcl pH8.0 buffer, at flow rate (36ml/hr).[18].

In vitro cytotoxicity

Neutral red assay was used in this assay [19]. Ninety-six well tissue culture plates used, a 50µl of extract added in the first and second wells of the first four lines. Two fold serial dilutions were made from the second well for the four lines that contain 50µl tissue culture medium (RPMI-1640) till the twelfth well in duplicate. The wells of the late four lines in the same plate were filled with 200 µl of tissue culture medium considered as a control. A 150 µl of RD cell suspension (10^4 cell / ml) was added to all wells of the plate. The culture plates were incubated at 37C in humidified incubator. The culturing plate was removed after 72 h, and 50µl of 0.01% of neutral red solution added to each well, reincubated for 2h, after incubation the medium was discarded and the wells washed with PBS. The results recorded as following; viable cells cells will take the dye, while the dead cells will not. 100µl of phosphate buffered ethanol (0.1M NaH_2PO_4-ethanol, 1:1) was added to each well to elute the dye from the viable cells. The plate was read by ELISA reder at optical density 492nm.

Results and discussion

In this investigation, an M-9 medium was used for L-asparaginase producing from local isolate of *E. coli*. In this medium, L-asparagine is used as the sole nitrogen source. The production of L-asparaginase by *E. coli* leads to ammonia formation and increase in pH of the medium. A pH indicator (phenol red) makes medium pink around the colonies producing L-asparaginase, (Figure 4).

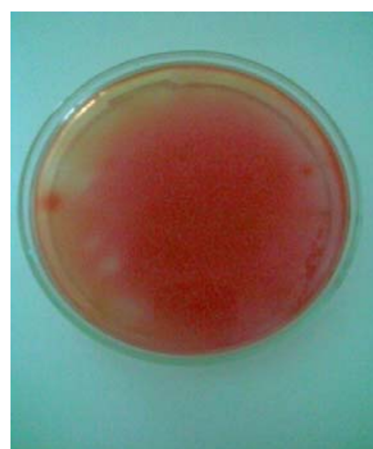

Figure 4: color pink for cultivating E. coli on M9 medium.

Standard curve of gradual concentration of ammonia liberated was used for quantitative measurement of L-asparaginase activity, which gives yellow color in the reaction. L-asparaginase specific activity was 0.05 U/mg in crude extract of the cells (Table 1). Roberts and his colleagues [13] showed that *E.coli* asparaginase specific activity was 0.08U/mg while Cedar and Schwarts get 3.4 u/mg when grown in other medium, so different activities were showed in different studies depend on the strain and culture medium [15].

Table 1: Purification profile of L-asparaginase from E. coli.

Purification Step	Volume (ml)	Enzyme Activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Yield (%)	purification fold
Crude	10	0.139	2.62	0.053	1.390	100	1
Dialysis	17	0.059	1.64	0.035	1.003	72	0.66
Ion exchange	14	0.041	0.23	0.18	0.574	41	3.39

Several methods for extraction were used cell lysis using lysozyme and EDTA [15] and homogenization [21] using homogenizer and spheroblast formation [22]. Sonication were used mostly [23, 18, 13] its simple and rapid method since sphere-oblast formation its good but needs an optimization for spheroblast formation. Liu. Zajic [24] extract 95% of the enzyme from *Erwinia aroideae* by sonication. In this work table (1) the specific activity using sonication was 4.18U/mg, and the protein concentration 0.03mg/ml, other studies showed that the specific activity from other strain of *E. coli* was 1.52 and protein concentration 1.2 mg/ml [17].

DEAE ion exchange was used because it has high resolving power and high protein-binding capacity and easy [25] and remove endotoxins from separated proteins and separate periplasmic L-asparaginase I from cytoplasmic L-asparaginase II [13]. DEAE ion exchange give two major peaks at fraction numbers 32 and 61. Fractions 58 to 64 were pooled and tested for L-asparaginase qualitative and quantitative activity (Figure 5) and cytotoxicity against RD cell line *in vitro.*

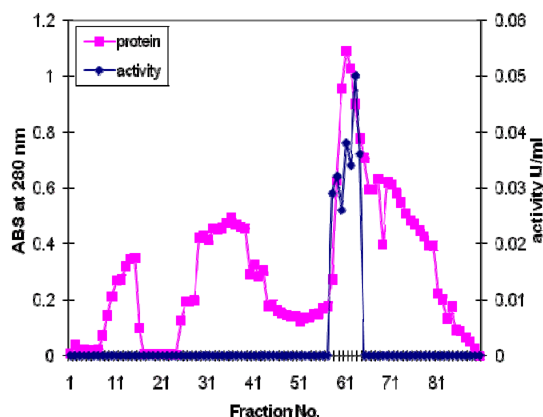

Figure 5: Ion exchange chromatography for L-asparaginase isolated from E. coli.

Purification of L-asparaginase from *E. coli* was achieved by using dialysis and DEAE-Cellulose Ion exchange chromato-graphy. Using this purification steps the specific activity was increased to 0.18 U/mg while the yield was decreased to 41% the number of folded was 3.39.

Cytotoxicity assay of crude extract and ion exchange purified factions was determined against RD cell line *in vitro.* L-asparaginase fractions showed slight inhibition percentage against RD cell line 16 and 17% inhibition (table (2)). Studies of the antitumor activity of the *L-asparaginase* usually done against lymphoma 6C3HED in C3H mice [13].

Table 2: The inhibition percentage ofM L-asparaginase extracts and control.

Sample	Inhibition%
Crude extract	**16**
Ion-exchange elute	**17**
Control	**0**

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