

Isolation, Partial Purification and Characterization of L-Asparaginase from Hedgehog Serum

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Abstract

The need to further explore other sources of therapy for leukemias prompted this current work. It's already known that L-asparaginase possess a lot of potentials in the treatment of cancers of the white blood cell but the sources of these enzyme and its properties determines the efficacy of this drug. Generally, for an asparaginase to be ideally suited for use in anti-tumour therapy, it had to satisfy a variety of criteria. The organism that is selected should produce the asparaginase in high quantity or yield, and it should be capable of being isolated from simple mammalian sources. The procedures developed for purification of the enzyme should be as rapid and simplified as possible. The purified enzyme should have long term stability on storage, maximal activity at a physiological pH, and a Km for substrate below the concentration of the substrate in the blood. Therefore the aim of this present research was to partially purify and characterize this enzyme from Hedgehog serum in order to compare its anti-leukemic potentials with those from documented literature for possible future medical application. L-asparaginase was isolated and partially purified from Hedgehog serum which is also known to be a reservoir of many other useful proteins including anti venoms using a four step profile of ammonium sulphate fractionation, dialysis, and ion exchange and gel filtration chromatography. The enzyme gave an overall yield of 77.58%, optimum pH and temperature of 7.8 and 39°C respectively, and a Km of 0.0125 mM. Gel filtration gave an approximate molecular weight of 139,000 Da, while SDS PAGE indicated a subunit molecular weight of 36,000 and 34,600 Da respectively. The enzyme also catalyses the hydrolysis of glutamine though slightly. Mg²⁺ and Zn²⁺ serve as activators for Hedgehog serum L-asparaginase, while inhibition from heavy ions like Hg²⁺ was also observed. This work shows that mammalian sources of this enzyme could be more preferable for treatment of asparagine dependent tumours in time to come.

Keywords: L-asparaginase; Tumor therapy; Leukemia; Hedgehog

Introduction

L-asparaginases are enzymes that catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia. They are also known as L-asparagine amido hydrolases (E.C. 3.5.1.1). The enzymes are present in many animal tissues, bacteria, plants, and in the serum of certain rodents and lower mammals but not of man. Asparagine is one of the twenty common natural amino acids on earth. It is a non-essential amino acid, since it can be synthesized by the body cells. Increasing interest in these enzymes stemmed from their identification by Broome in 1961 as anti leukemic agents in guinea pig serum. Following the discovery that the E-coli enzyme also had the anti-tumour activity by Mashburn and Wriston, work then preceded in several laboratories and other research centres leading to several clinical trials (Abuchowski et al.). L-asparaginases have been isolated from a number of sources which include; *Erwinia Carotovora*, *Serratia Marcescens*, and a host of other microbial and mammalian sources. Though the activity of this enzyme has been reported in many lower mammals, no such literature has been found for hedgehog serum till date.

L-asparaginases from *Erwinia* and *E. coli* have been reported for many years as effective drugs in the treatment of acute lymphoblastic leukemia (certain type of cancer). The Drugs are not however devoid of some serious side effects. Therefore, finding safer and cheaper sources of these enzyme is of paramount importance. L-asparaginases from bacterial species have been known to cause adverse allergic reactions, anaphylaxis and hypersensitivity in the long term, despite their high therapeutic (anti-tumour) properties. However, these immunological problems highlighted above can be circumvented by sequential therapy with serologically unrelated asparaginases [1]. This has prompted further research with a view to finding a variety of asparaginases from

other local sources like fungi, basidiomycetes, and small mammals like hedgehogs. Most of the Microbial sources of L-asparaginases are not amenable to isolation without the presence of so many interfering proteins thereby limiting the amount of useful enzyme preparation required for industrial/medical applications.

Mammalian and other fungal sources of L-asparaginases have been studied less thoroughly, as such more work is required on them and their anti-tumour potentials. There is also need to screen the enzyme from these mammalian sources in order to compare their properties with those documented in literature. In addition, producing mammalian L-asparaginases from sources similar to those of guinea pigs (with good anti-tumour activity) is highly required as a target for future cancer therapy. L-asparagine plays a very significant role in the metabolism of leukemic (tumour) cells. This is because, these cells require asparagines for protein biosynthesis and therefore when such cells are starved of asparagines, they get destroyed automatically. In addition, most cells are known to use the enzyme asparagines synthetase to make their own asparagines [2]. While most cells are able to make their own supplies of asparagines and do not need to obtain it in their diet, some blood

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cells, however, rely instead on the blood for their supply. This is the whole aim of L-asparaginase therapy considering the fast rate of cell division occurring in cancerous cells. In an attempt to address some of the salient issues raised above, the aim of this work is to extract the enzyme from a mammalian source so as to determine if they actually possess L-asparaginase activity when compared to those documented in literature and also to partially purify and characterize the enzyme from Hedgehog serum so as to find its anti-tumour potentials for possible future clinical trials.

Materials and Method

Hedgehog serum

Hedgehogs were hunted from the wild within Jaji-Igabi Local Government Area of Kaduna state. They were then slaughtered from the jugular vein and fresh blood collected into already prepared eppendorf tubes and allowed to clot on their own. Serum was subsequently taken into the Lab for further analysis.

Determination of L-asparaginase activity

Enzyme activity was determined using the method of Majundar et al. [3] as modified by the "Sigma quality control test procedure." The assay system consisted of 0.1 ml of enzyme extract, 0.1 ml of 0.01 M of L-asparagine, and 1 ml of 0.05 M tris HCl buffer. All these were pipetted into a test tube and placed in a water bath maintained at 37°C for 30 minutes. After the incubation, 1.5 ml of Tri Chloro acetic acid (TCA) was added to stop the reaction. These were then centrifuged for 2 minutes and the supernatant collected in a clean tube. Ammonia released by the catalysis of L-asparagine was estimated by Nessler's method. To 1 ml of the supernatant, 4.3 ml of distilled water and 0.5 ml of Nessler's reagent were added. This mixture was allowed to stay for another 2 minutes in a water bath at the same temperature. The colour intensity was measured at 436 nm using a Unicam Alpha spectrophotometer. Absorption was then compared to a standard curve of ammonium sulphate earlier determined in order to find the concentration of ammonia released. One enzyme unit (1 U) is defined as the amount of enzyme required to liberate one micromole of ammonia per minutes at 37°C under specified condition. Total protein was determined by Biuret method according to Plummer using Bovine serum Albumin.

$$\text{units / mg} = \frac{\mu \text{mole of } NH_3 \text{ released}}{\text{Time of reaction} \times \text{mg of enzyme in reaction}}$$

Enzyme purification assay

The purification assay for the enzyme was according to Borek and Lubkowski [4]. The following purification steps were carried out.

Ammonium sulphate fractionation/dialysis

The crude enzyme extract was precipitated out using different levels of ammonium sulphate saturation between 35-80%. The pellets were collected after each saturation interval by centrifugation at 10,000 g for 20 minutes at 4°C, and suspended in 0.01 M phosphate buffer pH 8.5. At each of this stage, enzyme activity and protein concentration determined. The dissolved pellets were further subjected to dialysis for 48 hours in a dialysis bag kept at 4°C and then loaded onto DE-52 ion

exchanger column (20 x 25 cm).

DEAE cellulose chromatography (ion exchange chromatography)/gel filtration chromatography using sephadex G-75

DEAE cellulose chromatographic column pre-equilibrated with 0.01 M tris HCl (pH 8.6) containing 0.01 M KCl was loaded onto a column (1.5 x 20) with 5 ml of the crude enzyme and the flow monitored and fractions collected. The column was washed with 5-10 volume of 0.01 M tris HCl + 0.5 M KCl and eluted with 0.01 M tris HCl. About 22 fractions were collected and assayed for L-asparaginase activity. Also, gel filtration chromatography with sephadex G-75 was performed for further purification and also to determine molecular weight. The column was eluted with the same buffer. All the purification was monitored by UV-Spectrophotometer.

SDS-page purification

Fractions containing high specific activity were pooled together and subsequently subjected to further purification using SDS-PAGE. These fractions were loaded onto a column packed with sephadex G-75 pre-equilibrated with 0.05 M phosphate buffer (pH 6.8) and the active eluates analyzed on 12% reducing SDS-PAGE. They were thereafter treated by washing with bromophenol blue and visualized with comassie brilliant blue. Gels were stained together with standard protein markers for molecular weight determination. After the electrophoresis, relative mobilities were calculated for each of the proteins. Relative mobilities (Rf) were obtained by measuring the distance migrated by the tracker dye. The molecular weight of the partially purified enzyme was calculated by proportion.

Optimum temperature

Activity measurements of the enzyme were carried at different temperature (0°C, 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, etc.). The enzyme activity was determined as described in section 3.2.0 at these temperatures and the rate of activity was plotted against temperature so as to find the optimal temperature.

Optimum pH

The optimum pH determination of the enzyme was carried out in different buffers with varying pH range (3.5-9.0). The enzyme activity rate at each pH was recorded and a plot of pH against enzyme activity was made to determine the optimum pH with varying L-asparagine.

K_m and V_{max} determination

Different concentrations of the substrate L-asparagine were prepared in the following order (2.5 mM-10 mM). The absorbances were determined at each of the above concentrations of the substrate at 436 nm. Thereafter, inverse of the activity rate was plotted against substrate concentration in order to determine the K_m and V_{max} respectively.

Effect of cations on enzyme activity

About 1.0 ml of the enzyme and 0.2 ml of 0.02 M of these cations (MgCl₂, KCl₂, CaCl₂, FeCl₂, and HgCl₂) were incubated at 37°C for 3-5 minutes and the activity determined as described before.

Effect of inhibitors on L-asparaginase activity

L-aspartate, L-glutamate, and L-Norvaline (specific inhibitor)

were used as inhibitors in this work. Aliquots of the enzyme(1 ml) and 0.2 ml of varying concentration of the inhibitors were incubated with substrate of varying concentrations(0.01-0.12 M).The activity were determined as previously described.

Results and Discussion

The effect of temperature on L- asparaginase activity is shown in Figure 1.Optimum temperature was recorded at 39°C. This is principally attributed to the fact that L-asparaginases in most cases act within the normal temperature range of man [5]. In addition, most medically active asparaginases possess optimum temperature between 35-40°C [6]. Generally, the effect of temperature on Enzyme-catalyzed reactions

is extremely complex. This is because a change in the observed rate may be due to a variety of causes like stability of the enzyme protein, pH of the buffer system, kM, and a host of other factors. The effect of pH on enzyme activity is shown in Figure 2. The pH optimum for the partially purified Hedgehog serum L-asparaginase is 7.8. This is in agreement with a number of pH optimums from various L-asparaginases from diverse sources (Table 1). Most L-asparaginases having antitumour activities have optimum pH's of between 7.5-8.5 as reported by Swain et al. [7]. Another reason for these observed optimum pH values are due to the fact that medically useful asparaginases normally act within physiological pH, i.e., around the normal pH of blood. Effect of substrate Concentration on enzyme activity is shown in Figure 1.

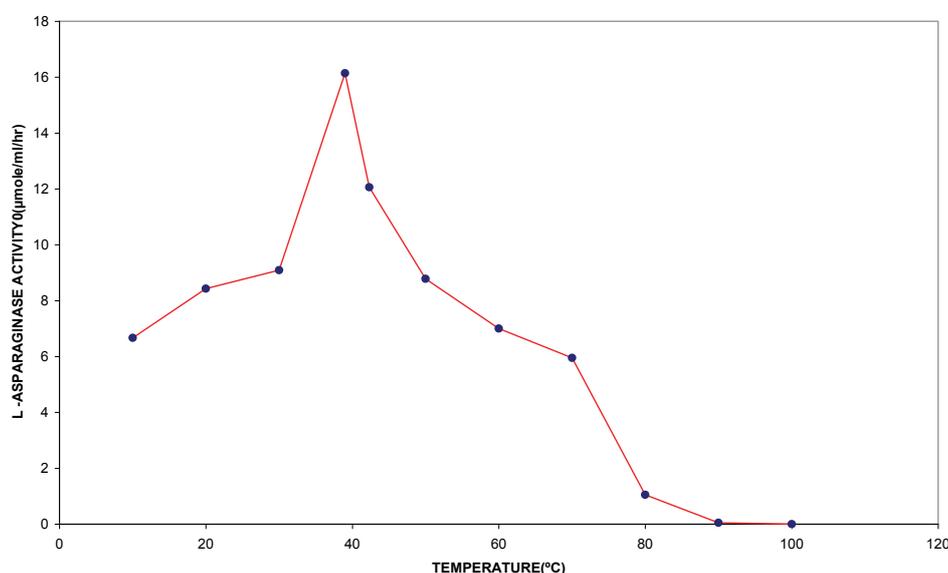


Figure 1: Effect of temperature on L- asparaginase activity.

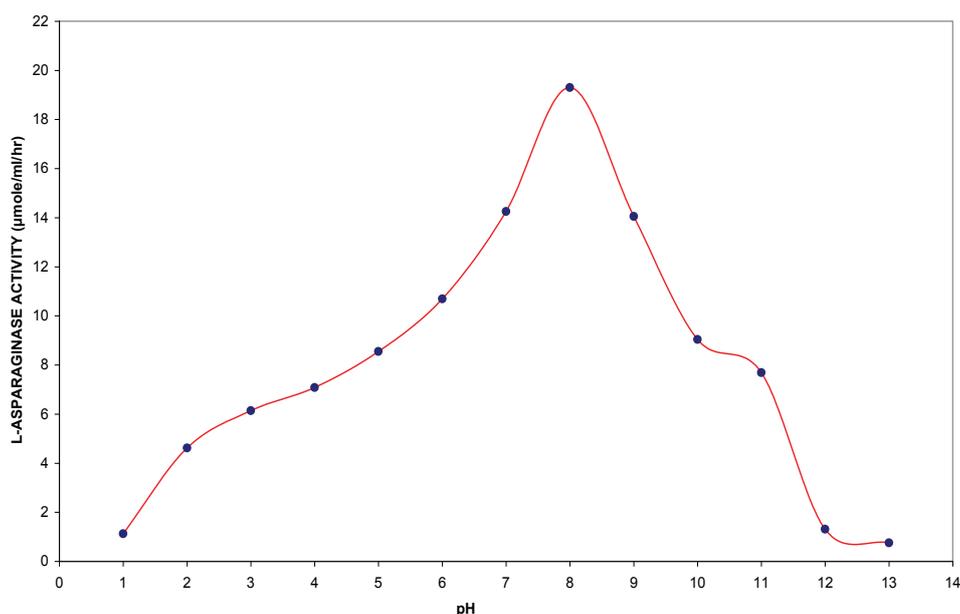


Figure 2: Effect of pH on L-asparaginase activity.

The highest activity was recorded at a substrate concentration of 0.01 mM. When the Lineweaver Burk's plot of the determination was done (Figure 2), Km and Vmax were gotten as 0.0125 mM and 1.6 mole/M respectively. These values are in line with those of other very useful chemotherapeutic L-asparaginases. Generally, asparagine does not become severely limiting for protein synthesis until the concentration is reduced to 0.01 mM or below and consequently, for asparaginases to be an effective antitumour agent, the enzyme must have a high substrate affinity. The effect of divalent cations is shown in Figure 2. Metals generally serve some specific roles in enzyme chemistry, they aid in the catalytic efficiency of the enzymes in which they are found (Co-factors). In this experiment, MgCl₂, ZnCl₂ and CaCl₂ were found to have some activating functions in the activity of Hedgehog serum L-asparaginase. This is in consonance with many asparaginases from bacterial sources [8-11]. HgCl₂ and FeCl₂ being heavy metals showed deactivating functions as expected with most heavy metals serving as co-factors.

Elution profile of partially purified Hedgehog serum L-asparaginase is shown in Figure 3. Two very conspicuous peaks were observed. The first major peak of fractions 4-6 gave specific activity of 42.78 μmole/m/hr/mg. Second peak (Fraction 7-10) gave almost similar values like the first suggesting that the DEAE cellulose-52 purification step was not conclusive enough. In all, about 17 fractions were collected and fractions with higher specific activities were collected, pooled together and ran with a sephadex G-75. Elution using sephadex G-75 gave a specific activity of 84.76 μmole /ml/hr/mg (Table 2, Figure 2). The purification fold rose up to 5.61 and yield was recorded as 61.00%. About 24 fractions were collected for the gel filtration chromatography. When these purifications were compared with those of other screened organism including *E. coli* (which served as reference). It was observed that Hedgehog serum L-asparaginase had a better yield of enzyme than all of them. (Table 1 and 2). This might not be far from the simple fact that mammalian sources of asparaginases possess higher yield in terms of quantity typified by yield from Guinea pig serum. Also, Hedgehog

	TOTAL PROTEIN (mg/ml)	TOTAL ACTIVITY (μmole/ml/hr)	SPECIFIC ACTIVITY (μmole/ml/hr/mg protein)	PURIFICATION FOLD	YIELD (%)
Crude	1.14	14.01	12.29	1.00	100
(NH ₄) ₂ SO ₄ Precipitation	1.05	13.63	12.98	1.06	97.29
Dialysis	0.75	12.87	16.93	1.38	91.86
DEAE-52	0.23	12.04	52.35	4.26	85.94
SEPHADEX G-75	0.15	11.24	74.93	6.10	80.23

Table 1: Purification profile of partially purified L-asparaginase from Hedgehog serum.

Source	Mol wt.	Subunits	pH optimum	Km (mM)	Glutaminase activity	Blood clearance time(hrs)	Anti-tumour activity
Guinea pig serum	138,000 133,000	N.D	7.5-8.5	0.072	Nil	26	+
<i>E. coli</i>	130,000	4	4.9	0.013	Slight	4.2	+
<i>Fusarium trinctum</i>	165,000	N.D	5.18	0.012	Nil	Very rapid	-
Green chillies	120,000	N.D	8.5	3.3	Yes	4	+
Hedgehog serum*	139,000 34,600	2	7.8	0.0125	Slight	9.8-16.4	+

Table 2: Properties of L-asparaginase having anti-tumour activity from different sources as compared with that from hedgehog serum.

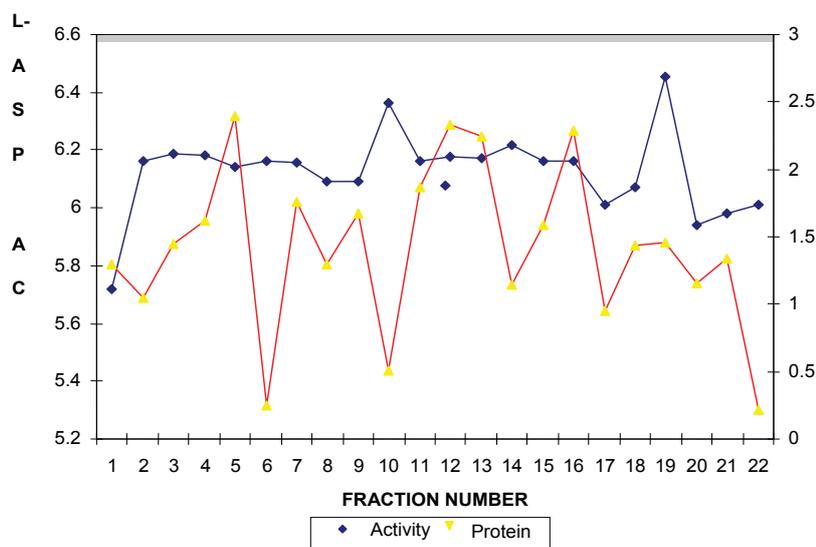


Figure 3: Elution profile of crude hedgehog serum L-asparaginase.

serum is known to be a reservoir of many useful proteins including anti venoms probably due to the harsh kind of habitat where Hedgehogs normally occupy in the ecosystem. Molecular weight determinations using the sephadex G-50 gave an average value of 139,000 Da for the partially purified Hegehog L-asparaginase. While SDS PAGE indicated subunits of 34.6 KDa and 36 KDa respectively (Figure 4 and 5). These values are consistent with most tumour inhibitory asparaginases with molecular weight between 120,000 and 150,000 Da. Molecular weight variations is one of the characteristic of active anti-tumour L-asparaginases. Furthermore, this molecular weight is very close to that of the Guinea pig serum L-asparaginase giving credence to the

assertion that serum from lower mammals could be one of the future target sources of this important enzyme.

Generally, for an asparaginase to be ideally suited for use in antineoplastic therapy, it had to satisfy a variety of criteria. The organism that is selected should produce the asparaginase in high quantity or yield, and it should be capable of being grown in large amount on a simple and inexpensive medium if it's a microorganism. The procedures developed for purification of the enzyme should be as rapid and simplified as possible, providing pure enzyme in high yield. The purified enzyme should have long term stability on storage, maximal activity at a physiological pH, and a Km for substrate below

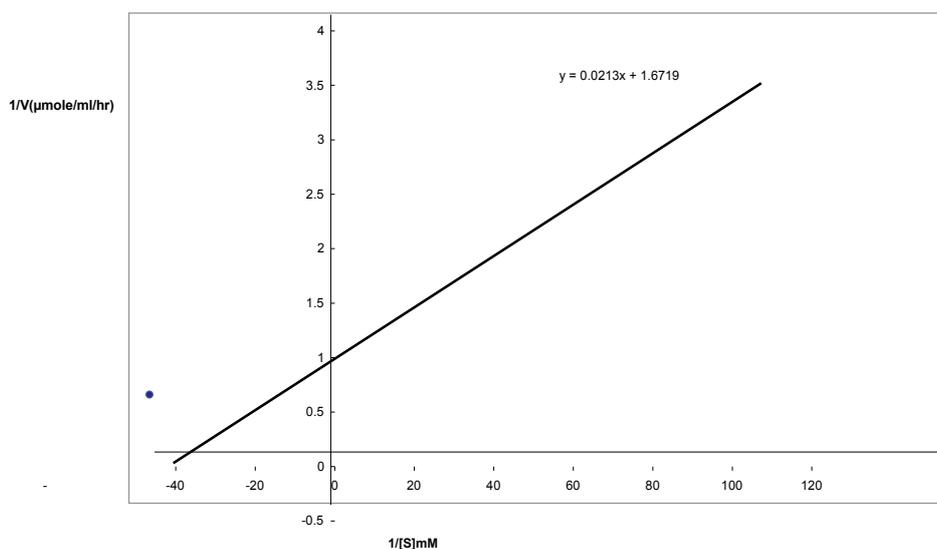


Figure 4: Line weaver burk's plot for hedgehog L-asparaginase activity.

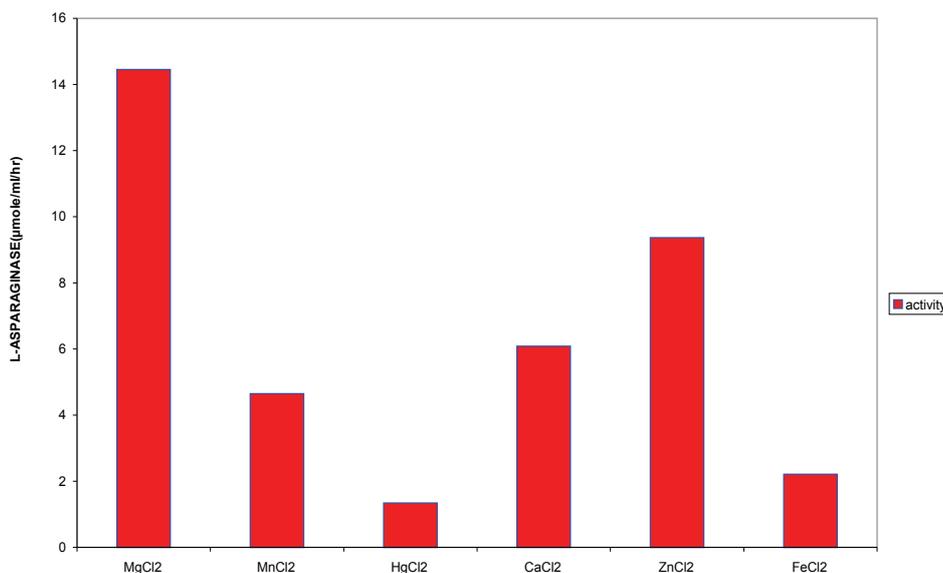


Figure 5: Effect of cations on L-asparaginase activity.

the concentration of the substrate in the blood. In conclusion, this work has shown that mammalian sources of this enzyme could serve as great therapy for asparagine dependent tumours.

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