Research Article

Purification and characterization of L-asparaginase from *Salinicoccus* sp. M KJ997975

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Abstract

L-asparaginase has been a major research subject worldwide because of its use as effective therapeutic agent against lymphocytic leukemia and lymphosacoma. Apart from its medical use it finds applications in food industry as well. The objective of the study was to purify, characterize L-asparaginase extracted from *Salinicoccus* sp. M KJ997975. The enzyme was purified to near homogeneity by ammonium sulphate precipitation (40-60%), followed by dialysis, gel filtration on Sephadex G-75 column and DEAE Cellulose A-50 ion exchange column. The enzyme was purified to 64 folds and showed specific activity of 105 IU/mg of protein with 3.55 % recovery. SDS-PAGE and gel filtration of the purified enzyme suggested that the protein may be a homotetramer of approximately 56 kDa with each subunit of approximately 14 kDa. The purified enzyme showed maximum activity at 37°C and pH 7 after incubation period of 30 minutes. Mg^{2+}, Ca^{2+}, K^{+} activated the enzyme whereas EDTA and Hg^{2+} inactivated it.

Keywords: Antileuckemic agent, L-asparaginase, Sephadex G-75, microbial flora, *Salinicoccus* sp.

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INTRODUCTION

The soil constitutes a conducive habitat for microorganisms and is inhabited by a wide range of microorganisms like bacteria, fungi, algae, viruses and protozoa. Microbes in the soil are important for soil fertility, biogeochemical cycles and various industrial products such as enzymes, antibiotics, vitamins, hormones, organic acids etc. L-asparaginase (L-asparagine amino hydrolase, E.C. 3.5, 11, LA) catalyses the hydrolysis of L asparagine into L-aspartic acid and ammonia. LA is broadly distributed among the plants, animals and microorganisms. Out of all microbes are considered as better source of this enzyme, because they can be cultured easily and its extraction and purification is also easy for large scale production[1]. Its chemotherapeutic potential in treating acute lymphoblastic leukemia and lymphosacoma has been well documented. LA treatment for ALL is a major breakthrough in modern oncology as it induces complete remissions in over 90% children within four weeks[2]. ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are some of the brands of LA, approved by FDA for the treatment of acute lymphoblastic leukemia and lymphosarcoma[3]. The purification of the protein is an important step in studying their physical and biological properties. Proteins used for therapeutic purposes need to have high degree of purity. Extra-cellular L-asparaginase are better than intracellular as they can be produced abundantly in the normal conditions and purified economically[4]. Wide range of microorganisms as its source, cost-effective production and eco-friendly nature has attracted considerable attention of LA worldwide. Still there is tremendous scope in screening of novel LA sources, studying their properties and applications in an attempt to find a production source that is abundant and commercially viable. In this study an attempt was made for purification characterization of L-asparaginase produced from *Salinicoccus* sp. M KJ997975 isolated from forest soil of Tungareshwar, Vasai, Maharashtra.

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MATERIALS AND METHODS

Chemicals

All the chemicals were of high analytical grade and procured from Hi-media, Sigma Aldrich and Merck. Solvents were procured from SRL, S.D. Fine.

Microorganisms

The organism used in the study was isolated from forest soil of Tungareshwar, Vasai, Maharashtra. The organism was identified as *Salinicoccus* sp. M KJ997975 by 16S rRNA sequencing[5].

Enzyme purification[6]

The extracellular L-asparaginase produced by this organism was then purified and characterized. The crude enzyme was purified first by ammonium sulfate precipitation. Maximum LA activity was observed with the fraction precipitated at 60% saturation. The precipitate was collected by centrifugation at 10000 rpm for 20 min and dissolved in a minimal amount of 0.01M Tris- HCl buffer (pH 8.4) and dialyzed overnight against the same buffer at 4˚C with frequent change of buffer. The dialyzed sample was further purified by gel filtration using a Sephadex G-75 column (30cm x 2cm) that was pre equilibrated with 0.01 M Tris-HCl buffer (pH 8.4). The protein elution was done with 0.01M Tris-HCl buffer (pH8.4), at a flow rate of 1-2 ml per 30 min. The active fractions were pooled and dialysed overnight against same buffer at 4^0C. Further purification was done by DEAE cellulose A-50 chromatography**.** The concentrated enzyme solution was applied to column of DEAE cellulose A-50 (30cm x 2cm) that was pre equilibrated with a 0.01 M Tris-HCl buffer (pH8.4). The column was washed with two column volume of the same buffer and the adsorbed protein was eluted using a linear gradient of KCl (0.0M to 0.5M) in 0.01 M Tris-HCl buffer (pH 8.4) at a flow rate of 1-2 ml per 30 min. Purified LA was assayed by direct nesslerization method[7,8]. Protein concentration was determined[9]. Purification fold, specific activity, % recovery of purified enzyme was calculated. The molecular weight of the enzyme was determined by SDS PAGE and Native PAGE. The gel was stained with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water. Molecular weight of the isolated protein was determined by SDS PAGE and Native PAGE and compared to molecular weight markers[10].

Enzyme characterization[6]

Effect of pH, temperature and incubation time on purified LA

The activity of LA was assayed at different pH, temperature and incubation time. Purified enzyme was incubated with buffers of pH 4-12 under assay conditions and the amount of ammonia liberated was determined. The buffers used were potassium phosphate (pH 4.0-7.0), Tris-HCl (pH 8.0-9.0), and glycine-NaOH (pH10).Optimum temperature of enzyme activity was determined by incubating the assay mixture at temperatures ranging from 4 to 60^0C.The effect of the incubation time on L-asparginase activity was studied in the range of 0 to 60 min. The enzyme characterization experiments were performed in triplicates.

Substrate Specificity and Determination of Km and Vmax

Identical reaction mixtures containing the same amount of enzyme preparation received an equimolar amount of a specific substrate namely L- asparagines and L-glutamine and were incubated under the standard assay conditions. The enzyme kinetics as measured by the Michaelis constant (Km) 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 (Km) value of the purified enzyme was estimated in a range of L-asparagine concentrations of 0.02 0.08 μM. The apparent Km and Vmax value of purified L-asparagine was calculated from the Lineweaver-Burk plot.

Effect of activators and inhibitors on enzyme activity

The effect different activators like Na^+, K^+, Ca^{2+} and Mg^{2+} at different concentrations (0.2-4 mg/ml) and inhibitors like EDTA, Hg^{2+} and Zn^{2+} at different concentrations (0.4-2mM) on the enzyme activity was tested after incubation with the purified enzyme for 2 h. After the exposure time, enzyme activity in each sample was assayed and expressed as a relative activity percentage calculated from the ratio of the specific activity of the treated LA to that of the untreated sample.

Statistical analysis

All the results were expressed as mean $\pm$ standard deviation (S.D.).

RESULTS

Purification of L-asparaginase

The partial purification of L-asparaginase crude extract that was affected by the ammonium sulphate (60%) precipitation showed that most of enzyme activity was preserved in the precipitate (Fig 1).

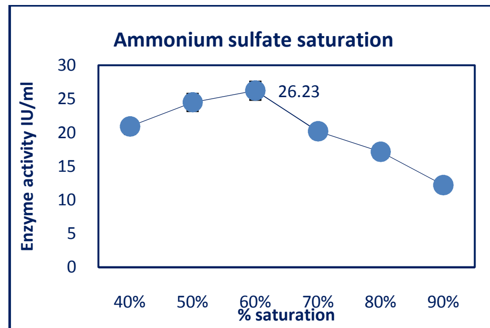

Figure 1: LA activity in IU/ml after ammonium sulfate precipitation at various % concentrations after 24 h of incubation at 4^0C.Values are expressed as mean ± S.D., with highest precipitation at 60% of ammonium sulfate precipitation

The total protein content decreased from 17.3 to 0.15 mg but the specific activity increased from 0.164 to 105 IU/mg, with approximately 10.66 fold purity by ammonium sulphate precipitation. Further purification of the enzyme by Sephadex G-75 resulted in specific activity of 57.7 IU/mg with approximately 35.18 folds purity and yield of 24.53 % (Fig 2).

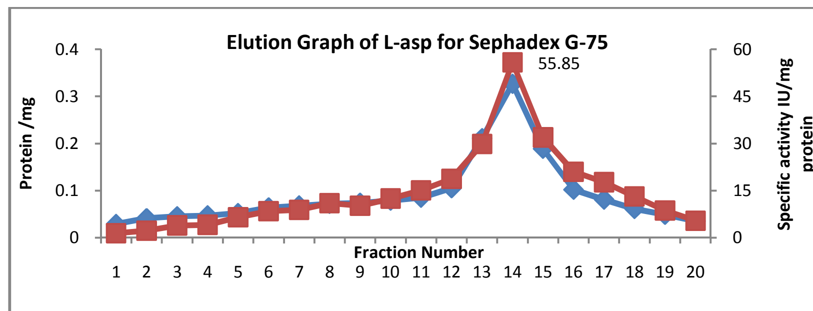

Figure 2: Elution profile of Gel filteration chromatography of LA. The dialyzed ammonium sulphate precipitate was chromatographed on Sephadex G-75. Total protein was monitored at 280 nm. The fractions were assayed for enzyme activity. Fraction no. 14 had highest specific activity of 55.85 IU/mg of protein

The final purification of L-asparaginase was achieved by DEAE cellulose A-50 column chromatography which resulted in specific activity of 105 IU/mg (Fig 3), approximately 64.02 folds purity, and yield of 3.55% (Table-1).

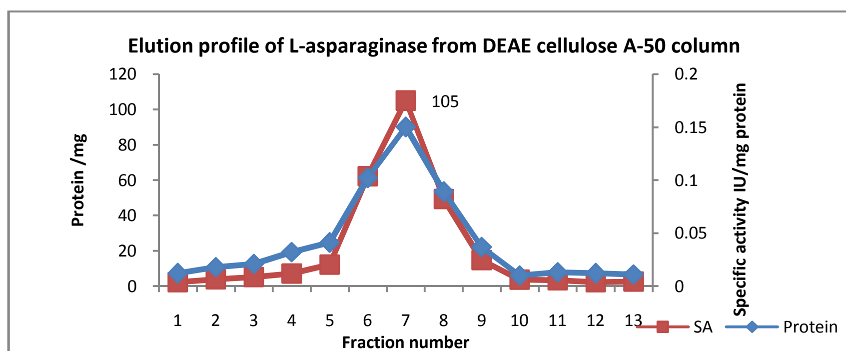

Figure 3: Elution profile of Ion exchange chromatography of LA. The active fractions collected, pooled after gel filtration were chromatographed on DEAE cellulose A-50 column. Total protein was monitored at 280 nm. The fractions were assayed for enzyme activity Fraction no. 7 with highest specific activity of 105 IU/mg of protein eluted with 0.3M of KCl

Table 1: Summary of Purification- showing the values of enzyme activity (IU/ml), Protein concentration (mg/ml), Specific activity (IU/mg of protein), Total activity (IU/ml), Fold purification and % yield of various stages of purification of LA from *Salinicoccus* sp. M KJ997975

Sample	Enzyme Activity (IU/ml)	Protein (mg/ml)	Specific activity (IU/mg of protein)	Total activity (IU/ml)	Fold Purification	% yield
Crude Extract	28.5	17.3	1.64	4275	1	100
60% Dialysis	26.24	1.5	17.49	1312	10.66	30.69
Gel Filtration	21.87	0.379	57.7	1094	35.18	24.53
DEAE ion exchange Chrom	15.75	0.15	105	151.76	64.02	3.55

Molecular weight of L-asparaginase

SDS-PAGE demonstrated the purity of the enzyme which moved as single band. By comparing the protein with commercially available standard protein molecular weight markers the apparent molecular weight of LA from *Salinicoccus* sp. M KJ997975 was found to be nearly 56 kDa, indicative of the protein being a homotertamer with each subunit of appox.14 kDa (Fig 4a, 4b) when compared to molecular weight markers.

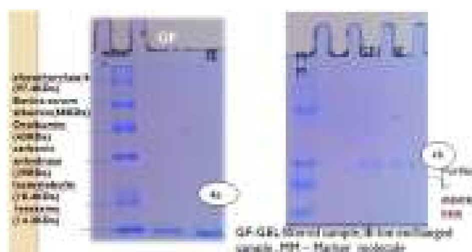

Figure 4a: Separation of protein bands through SDS PAGE

Figure 4b: Separation of protein bands through Native PAGE

Lane 1- Separated Bands of various protein molecular weight markers, Lane 2- Showing one band of purified LA from *Salinicoccus* sp. M KJ997975 obtained through sephadex G-75 chromatography separation with approx. molecular weight of protein subunit as 14 kDa, Lane 3- Showing one band of purified LA from *Salinicoccus* sp. M KJ997975 obtained through DEAE cellulose A-50 chromatography separation with approx. molecular weight of protein subunit as14 kDa. Lane 1: Separated bands of various protein molecular weight markers, Lane 2- Showing one band of purified LA from *Salinicoccus* sp. M KJ997975 obtained through sephadex G-75 chromatography separation with total molecular weight of protein as approx.56 kDa, Lane 3- Showing one band of purified LA from *Salinicoccus* sp. M KJ997975 obtained through DEAE cellulose A-50 chromatography separation with total molecular weight of approx.56 kDa.

Enzyme characterization

The influence of pH on the L-asparaginase activity was studied by using buffers of pH values ranging from 4-12. The enzyme activity gradually increased until pH 7 and then decreased (Fig.5). The effect of temperature on the stability of purified L-asparaginase was determined by incubation of enzyme preparation at different temperature in the range (4-60˚C) for 30 min. Maximum activity was obtained at an incubation temperature of 30˚C (Fig.6). The effect of incubation period on L-asparaginase was studied in the ranges of 0-60min (Fig.7). L-asparaginase activity increased as the incubation time increased, the activity was maximum for 30 min and decreased as the time increased. The activity of enzyme was also determined with different substrates like L-asparagine, L-glutamine, D-asparagine with 0.04 M concentration to find out the most specific substrate for the purified LA. It was found that the L- asparagine was most suitable substrate for the LA of *Salinicoccus* sp. M KJ997975. The purified enzyme exhibited 15.91 IU/ml of LA activity. Moreover, it did not exhibit glutaminase activity. Km and Vmax values of the purified LA as 0.02 M and 20.40 IU/ml of protein, respectively, and this indicates the high affinity of the enzyme to the substrate (Fig 8).Mg^{2+}, Ca^{2+}, K^{+} activated the enzyme. Mg^{2+} showed the specific activity of 141.69 IU/mg. EDTA, Hg^{2+} and Zn^{2+} decreased the enzyme activity (Fig. 9)

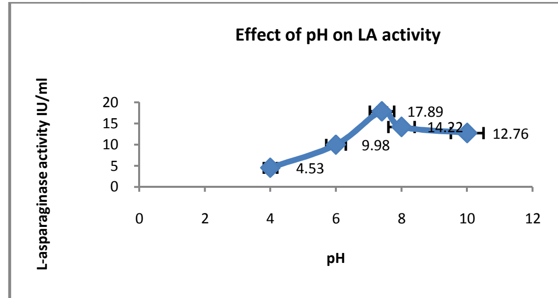

Figure 5: Effect of pH on purified LA activity studied in the range value from 4-10 using various buffers. Values of LA activity expressed as mean Mean + S.D.

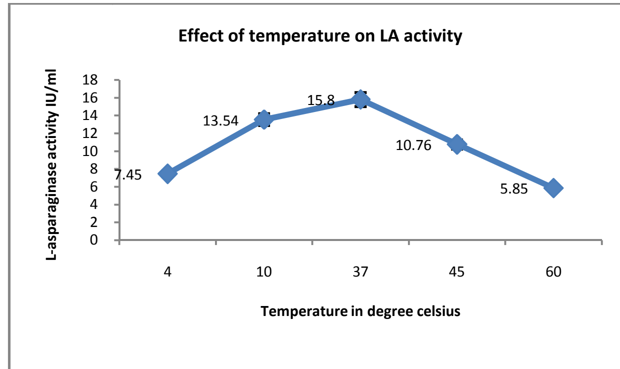

Figure 6: Effect of temperature on purified LA activity studied in the range of 4-60 ˚C in a Tris-HCl buffer (pH 8.0) after 30 min. of incubation. Values of LA activity expressed as mean Mean + S.D.

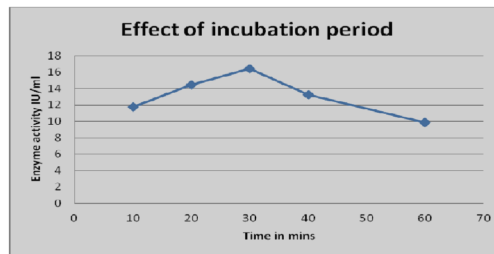

Figure 7: Effect of incubation time on purified LA activity studied in the range value of 10-60 min. in a Tris-HCl buffer (pH 8.0) at 30^{0} C. Values of LA activity expressed as Mean + S.D.

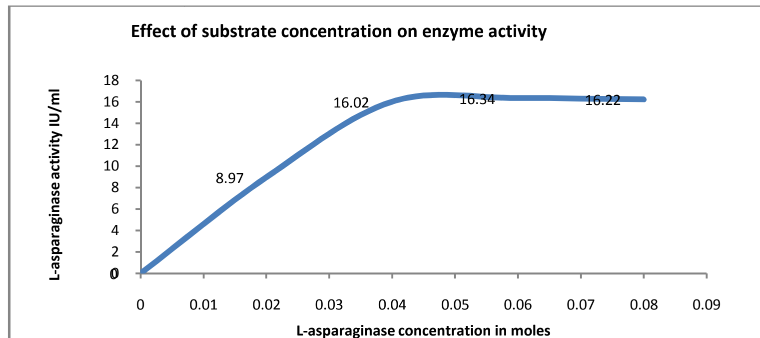

Figure 8: Effect of increasing substrate concentration (L-asparagine) in moles on purified LA activity

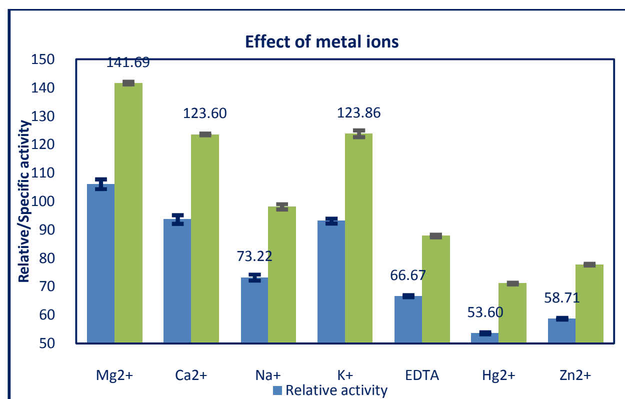

Figure 9: Effect of various metal ions on LA activity. Values are expressed as Mean ± S.D

DISCUSSION

LA (L-asparagine amidohydrolase, E.C.3.5.1.1) is a clinically acceptable anti-cancer agent, for the effective treatment of certain lymphomas and leukemias in both experimental animals and humans. It has been used in combination with other agents in the treatment of acute lymphoblastic leukemia (mainly in children), reticle sarcoma, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia and chronic lymphocytic leukemia. Enzyme purification is a multistep process which is necessary to study its structure, kinetics, mechanisms, regulations and role in a complex system. Purity of the enzyme is very important for medical and industrial applications**.** Purification of L-asparaginase from *Salinicoccus* sp. M KJ997975 was achieved by using 60% saturated ammonium sulphate precipitation, Sephedex G-75 gel filtration and DEAE cellulose A-50 anion exchange column chromatography. Nearly 64 fold purity of L-asparaginase was obtained in final purification step. Ultrasonication was used to destroy the *P. vulgaris* Pv.U.92 cells then ASNase II was extracted and purified throughout several purification steps including precipitation with $(NH4)_2SO_4$ (60-80%), DEAE-cellulose ion exchanger chromatography followed by Sephacryl S-300 filtration. The specific activity was 155.6 U/ mg and the purification fold was 27.3 with 10.4% yield[11]. The high amount of LA production was observed in optimal medium under 30 min incubation, pH 7 and temperature of 40^0C. LA was purified with ammonium sulfate precipitation[12]. The results of purification of LA from *Salinicoccus sp* M KJ997975 are in accordance with the literature cited. The purified enzyme was protein profiled by SDS-PAGE for determination of molecular weight of the enzyme which revealed a protein band with molecular weight of 56 kDa. which can be a homotertamer with each subunit of appox.14 kDa. (Figure 4a,4b). The molecular weight of LA from *Pseudomonas aeruginosa* was confirmed following the enzyme pattern analysis using SDS PAGE and found to be 39000 Daltons[12]. The apperant molecular weight of purified LA from *Serratia marcescens* was found to be 34 kDa[13]. Molecular weight of purified L-asparaginase obtained from *E. coli* VRY-15 was found to be 56 K Da as determined using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[14]. Purified LA from *E. coli* MTCC 739 was obtained in tetrameric form having a molecular mass of around 150 kDa[15]. SDS PAGE of purified LA from *E.coli* W3110 was found to be 40KDa[16]. Literature reveals that LA extracted from various sources differ in its molecular weight as well as it shows diversity in its subunit structure. Study of activators and inhibitors demonstrated that purified LA activity was inhibited by increasing concentration of EDTA followed by Cu^{2+} and Zn^{2+}. On the contrary increasing concentration of Mg^{2+} increased enzyme activity and maximum activity was observed with 4mg / ml of activator. Calcium and potassium also enhanced enzyme activity (Fig. 9). Inhibition of enzyme activity with EDTA possibly suggested that the purified LA might be a metaloenzyme[17]. Purified LA activity was strongly inhibited by EDTA followed by Cu^{2+}, Zn^{2+}, while stimulated by increasing concentration of Mg^{2+}, Ca^{2+}, K^{+}. The inhibition of LA from marine *Actinomycetes* by Cu^{2+} and EDTA has been reported[18]. Similar results were reported to be seen in LA from *Streptomyces noursei*. LA from *Pseudomonas aeruginosa* 50071 showed its maximum activity at 30 min of incubation[19]. Our results are also in agreement with reports for LA from various bacterial species[20,21]. LA shows diversity in its optimum pH, temperature, incubation period, activators and inhibitors as per the sources.

CONCLUSION

LA produced from *Salinicoccus* sp. M KJ99797 has promise as an antineoplastic agent. The properties of this enzyme such as optimal activity at pH 7 and at 30°C, makes it extremely valuable in chemotherapy. It is important to note that the purified LA from *Salinicoccus*

sp. M KJ99797 did not elicit any immunostimulatory response in normal human lymphocytes *in vitro*. However, further studies are needed to characterize the mode of action of LA from *Salinicoccus* sp. M KJ997975 *in vivo*.

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