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PURIFICATION AND CHARACTERIZATION OF L-ASPARGINASE ENZYME FROM SPINACEA OLERACEA

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ABSTRACT

L-asparaginase (E. C. 3.5.1.1) is an anticancer enzyme used as a drug to treat acute lymphoblastic leukaemia. In the present piece of work, L-asparaginase was purified from Spinacea oleraceae. The L-asparaginase enzyme was purified by salt precipitation (70%) followed by dialysis, anion exchange chromatography (DEAE- Cellulose) and gel filtration (sephadex G75) with final yield of 47.4% and purification fold 20.30. The molecular mass was found to be 160kda as estimated by SDS-PAGE. The enzyme activity of purified sample was found to be 322.3 IU and specific activity 1587.6µmoles/mg/min. This study aimed to determine novel source of L-asparaginase enzyme from Spinacea oleraceae, a possible solution for blood cancer.

KEYWORDS: L-asparaginase enzyme, Spinacea oleraceae, acute lymphoblastic leukaemia.

INTRODUCTION

L-asparaginase (E. C. 3.5.1.1) catalyzes the deamidation of L-asparaginase to produce L-aspartic acid and ammonia. This antileukemic L-asparaginase enzyme has been isolated from a number of microbial sources and their physicochemical properties have been studied extensively.[1,2]

Though L-asparaginase has been reported in many plants, little work has been carried out on the purification and characterization of this enzyme from higher plants. One such example is *Withania somnifera*[3] (Oza *et al* 2009). In plants, L-asparagine is the major nitrogen storage and transport compound and it may accumulate under stress conditions[4] (Sieciechowicz *et al*. 1988). The ammonia liberated from asparagine by the action of L-asparaginase enzyme is

necessary for protein synthesis. The L-asparaginase derived from Erwinia caratovora and E. coli have anticancer property and have been reported for many years as effective drug under the trade name Erwinase and Elspar for the treatment of acute lymphoblastic leukemia.[5,6] But their main side effects include anaphylaxis, pancreatitis, diabetes, leucopoenia, neurological seizures and coagulation abnormalities. Therefore an attempt has been made to find novel source of this enzyme from edible plants. Extraction and characterization of L-asparaginase from crude extract of Spinacea oleraceae has already been reported with good results[7] (Sarina *et al* 2016).

The present investigation describes the purification and characterization of L-asparaginase from spinacea oleracea.

MATERIALS AND METHOD

Purification of L-asparaginase enzyme

Salt Precipitation

The crude extract of Spinacea oleracea[7] was used for 70% ammonium sulphate saturation. Ammonium sulphate salt was added by stirring the solution continuously using a magnetic stirrer under ice cold condition. After complete addition of salt, it was refrigerated for an hour and then centrifuged at 10,000 rpm for 10min at 4°C. The pellet was resuspended in 10mM Tris Hcl of pH7. Total protein was estimated and enzyme assay was carried out. The resuspended solution was further used for the next purification step.

Dialysis

Activation of Dialysis membrane: 100ml of distilled water was boiled and dialysis membrane (8kb) of 8cm was placed into it and continued boiling for 10 minutes. 1g of sodium bicarbonate was added to it and boiled for 10 minutes. The solution was replaced with fresh water and boiled for 10 minutes with the membrane in it. The resuspended sample was transferred into the activated dialysis bag and the bag was sealed from both the sides without any air bubbles. The bag was kept in a plant tissue culture bottle, filled with distilled water (pH 7.0) and refrigerated overnight. After overnight refrigeration, the dialysis bag was then placed on the magnetic stirrer and subjected to stirring. The water was changed frequently for every half an hour to avoid establishment of equilibration. The dialysis was performed for about 3 hrs. Total protein was estimated and enzyme assay was carried out. The dialysed sample was used for the next purification step.

Ion Exchange Chromatography

Ion Exchange Chromatography relies on the attraction between oppositely charged particles. In the present study, anionic exchanger DEAE Cellulose column was used; the column attracts the negatively charged protein molecules and hence helps in purification. The stationary phase consisted of DEAE Cellulose and the mobile phase used was the elution buffer. The elution buffer consisted of five different concentrations of TrisHCl and Sodium chloride solution.

Table 1: Concentration of the mobile phase elution buffers

Elution buffer	Concentration of TrisHCl	Concentration of NaCl
Fraction 1	25mM	25Mm
Fraction 2	25 mM	50mM
Fraction 3	25 mM	75mM
Fraction 4	25 mM	100mM
Fraction 5	25 mM	125mM
Fraction 6	25 mM	150mM

The column was pre- processed by washing with methanol and then drying it. The column was then packed with DEAE cellulose (anion exchange A-50) of measurement 13mm radius and 150mm length. Before eluting samples, the column was washed by using distilled water twice and kept for sonication for 15 minutes. The matrix was then activated using the activation buffer (fraction 1). The dialyzed protein sample was transferred into column followed by the addition of elution buffer for elution of the protein. The process of elution was carried out using solutions of different fractions 2, 3, 4, 5, 6 respectively which contains different concentration of NaCl. Protein estimation was performed for the collected fractions by Lowry's Method [8]. Protein of mg/ml was calculated using BSA standard curve and enzyme assay was carried out after each purification step. The purified sample was then subjected to Gel filteration Chromatography.

Gel filteration /Size Exclusion Chromatography

The purified sample obtained after Ion exchange chromatography was subjected to Gel filtration column in which clean column was filled with Gel bead matrix (Sephadex G75). Sample was added after the matrix settled. 100mM Phosphate Buffer (pH 7.0) was used to stabilize the protein. Elutions were collected every 5 minutes. Protein estimation and enzyme assay was carried out for all the samples collected.

Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The separating gel consisting of 5ml distilled water, 3.5ml of 30% stock acryl amide, 2ml of separating buffer (pH 8.8), 100 µl of 10% SDS, 100 µl of 10% ammonium per sulphate and 15µl of TEMED was poured between the glass plates until three- fourth of the unit was filled and kept for 30 minutes without disturbing for polymerization. The stacking gel consisting of 3.5ml distilled water, 1.0ml of 30% Stock Acryl amide, 500µl Stacking buffer (pH 6.8), 100 µl of 10% SDS,50µl of 10% ammonium per sulphate and 10µl of TEMED was allowed to polymerize for about 20 minutes. Teflon comb was inserted into the stacking gel solution. The comb was removed carefully after polymerization. The wells were washed immediately with water and the excess water was removed by inverting the gel. The glass plate was fixed in the electrophoretic apparatus and the running gel buffer was filled in the tank. One part of sample was mixed with 1 part of loading dye. The mixture was boiled at 60˚C for 05 minutes. The samples were loaded into different wells. The gel was applied with 20mA and the power supply was switched off when the dye front reached the bottom of the gel.

Staining procedure: After electrophoresis, the gel was transferred to a clean plastic container. The stacking gel is removed completely form the separating gel, to which staining solution was added consisting of 40 ml methanol, 55 ml water, 5 ml acetic acid and 250 mg R-250 Coomassie brilliant blue. It was incubated overnight at room temperature with continual shaking in a Gel rocker. The gel was further de-stained by adding 40 ml methanol, 55 ml water, 5 ml acetic acid and allowed to stand for 1 hour. The bands were viewed under the trans-illuminator. The gel was stored in the gel storing solution of 10% acetic acid.

Enzyme assay

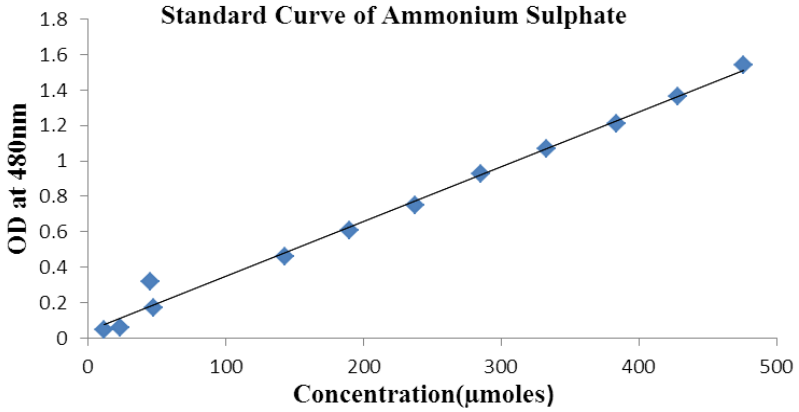
In a clean dry test tube 0.5ml of phosphate buffer of pH8, 0.5ml aspargine (substrate) and 0.5 ml enzyme (salting out sample, dialysate sample, ion exchange chromatography elute and gel permeation chromatography elute) was added. The volume was made up to 3.0ml with distilled water and incubated for 15 minutes at 37^0 C. 0.1 ml of 20%TCA and 1.0 ml Nessler's reagent was added and mixed well. The absorbance was measured at 460nm. A control was prepared without the enzyme sample.

Characterization of Protein

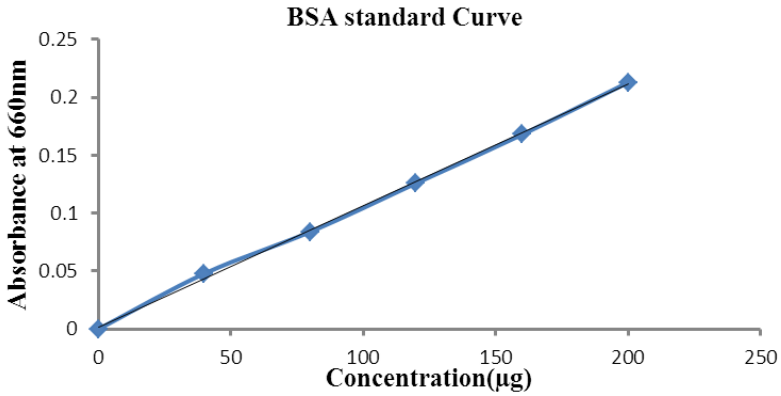
Temperature, pH and time kinetics was carried out. Km and Vmax values were also determined.[7]

RESULT

The leaf extract of Spinacea oleraceae used as crude enzyme source was purified by a series of purification steps that included salt precipitation, dialysis, anion exchange chromatography and gel filtration. Enzyme assay and protein estimation was carried out after each purification step. The purification fold increased after each purification step. The purified enzyme was subjected to SDS-PAGE and the molecular weight of L-asparaginase was found to be 160kda. Enzyme characterization of the purified sample was carried out and optimum conditions of L-asparaginase have been reported.



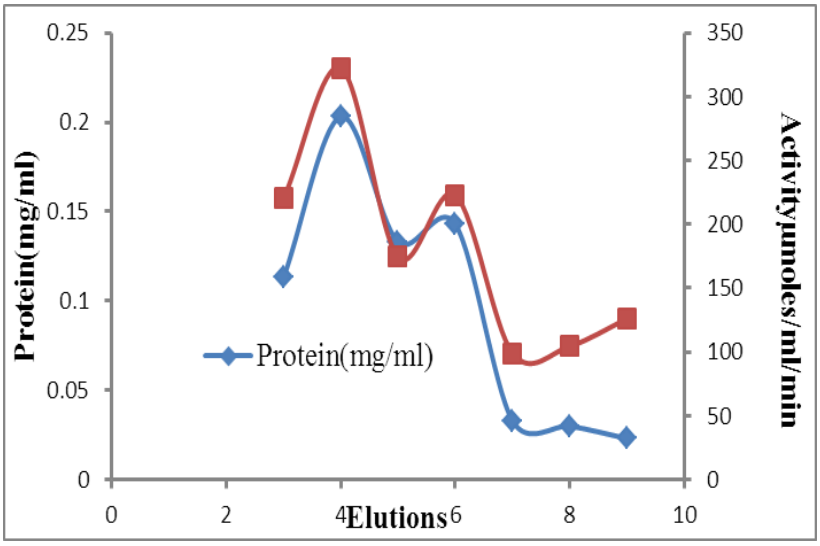
Graph 1: Ammonium sulphate standard graph to calculate the enzyme activity.



Graph 2: Lowry's standard graph to calculate the specific activity.

Table 2: Purification table showing purification fold and % recovery.

Sample	Activity (µmoles/min)	Protein mg/ml	Specific activity (units/mg)	Fold purification	Percentage of yield
Crude	679.84	8.693	78.20	1	100
salt precipitation	470.158	10.719	43.86	0.56	69.15
dialysis	281.98	9.249	30.48	0.38	41.47
Ion Exchange	392.2	6.363	61.63	0.788	57.69
Gel filtration	322.30	0.203	1587.6	20.30	47.40



Graph 3: graph representing the amount of protein and enzyme activity in each of the elution collected during the process of gel filteration.

SDS-PAGE

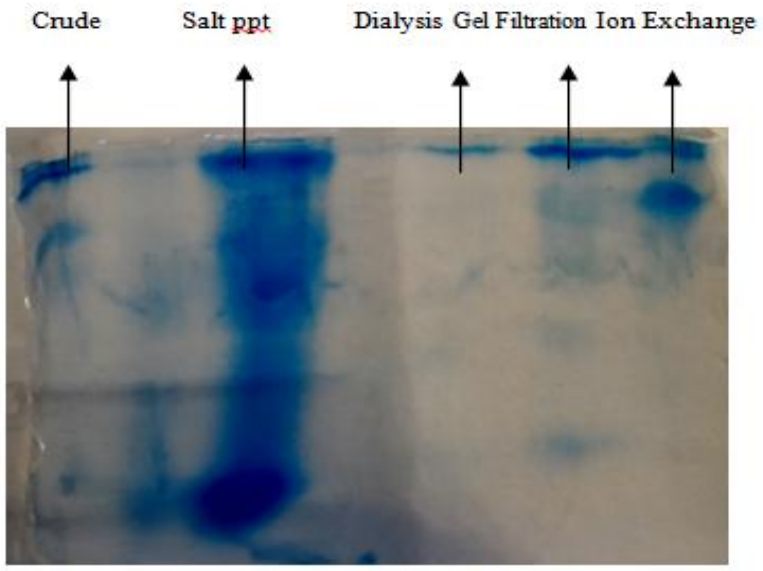
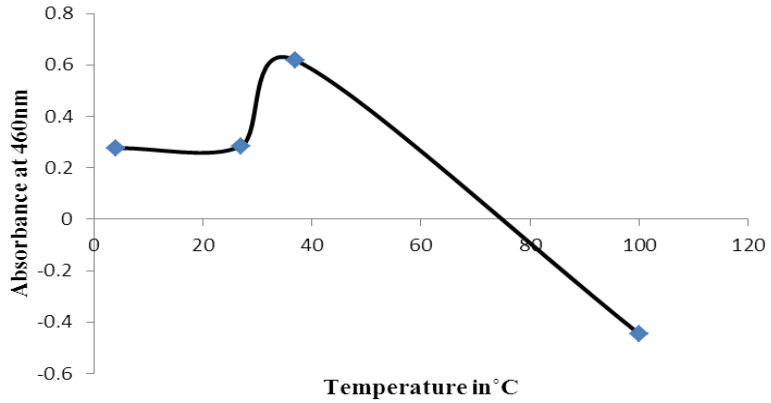


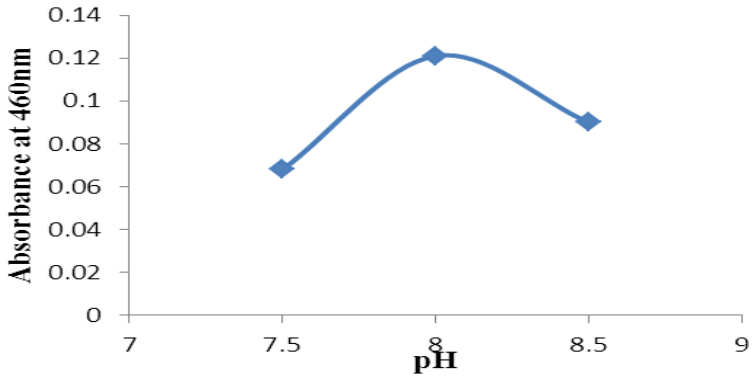
Figure 1: The samples of different purification step was subjected to SDS-PAGE to determine molecular weight.

Temperature Kinetics



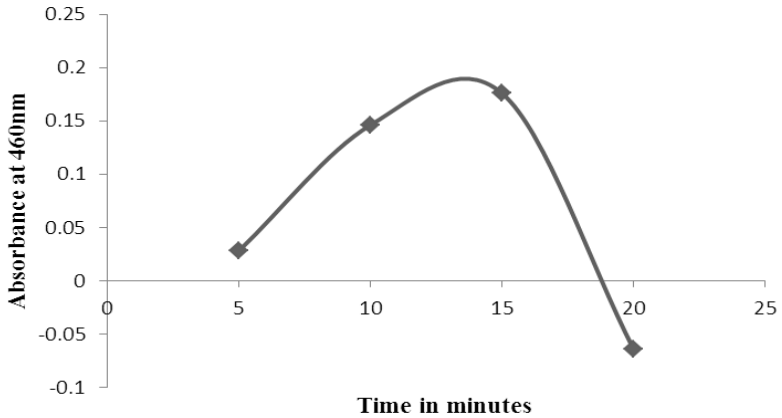
Graph4: The optimum temperature of L-asparaginase enzyme in *Spinacia oleracea* was found to be 37°C.

pH Kinetics



Graph5: The optimum pH of L-asparaginase enzyme in *Spinacia oleracea* was found to be pH 8.

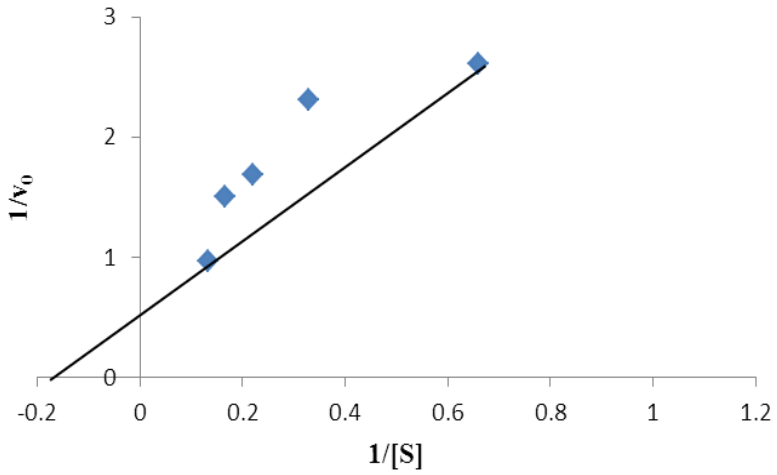
Time Kinetics



Graph6: The optimum time interval of L-asparaginase enzyme in *Spinacia oleracea* was found to be 15 minutes.

Table 3: Km and Vmax.

[S] in mg	1/[S]	V_o	1/ V_o
1.5	0.66	0.383	2.610
3	0.33	0.432	2.314
4.5	0.22	0.591	1.692
6	0.166	0.664	1.506
7.5	0.133	1.028	0.972



Graph 7: Km of Lasparaginase enzyme in *Spinacia oleracea* was found to be 5.5.

Vmax of L asparaginase enzyme in ***Spinacia oleracea*** was found to be 2.0µmoles/ml/min.

DISCUSSION

The leaf extract of spinacea oleraceae showed L-asparaginase enzyme activity and hence the crude extract was subjected to a series of purification steps. The enzyme activity of purified L-asparaginase enzyme was 322.30IU and specific activity 1587.6µmoles/mg/min. The final purification fold obtained was 20.30 and the percentage of yield was 47.4%. The Molecular weight of L-asparaginase enzyme was determined to be 160kda. The biochemical characterization of purified enzyme was carried out and the optimum conditions of the L-asparaginase enzyme with respect to temperature was 37˚C, pH was 8 and incubation time was 15min. The Km value of L-asparaginase enzyme from spinacea oleraceae was found to be 5.5 and Vmax was 2 µmoles/ml/min, by means of the double reciprocal Lineweaver- Burk plot.

CONCLUSION

Biochemical assay and characterization confirmed the production of L-asparaginase enzyme in Spinacea oleraceae. L-asparaginase enzyme showed similar kinetic specifications to the L-asparaginase enzymes reported earlier. It has been reported that the L-asparaginase drug from

microbial source cause major side effects, hence this enzyme from Spinacea oleraceae, would fight acute lymphoblastic leukemia more effectively because it is an edible herb.

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