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Extraction and Characterization of L-asparaginase from *Spinacea oleraceae*

Sarina P. Khabade*, Mahir S. Patel and Sowjanya R.
Department of PG studies and Research in Biotechnology, Government Science College, Bangalore-01, India
sarin28@rediffmail.com

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Abstract

L-asparaginase (E.C. 3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. It is identified as an effective antitumor agent in human clinical trials and is now recognized as one of the important component of antitumor therapy. The purpose of the present study was to screen for the production of L-asparaginase enzyme in Spinacea oleracea. The presence of the enzyme was confirmed by the formation of ammonia which was detected using UV-Visible Spectrophotometer at 460nm. The enzyme activity was found to be 1.9IU and specific activity was 6.8 µmoles/mg/min. The enzyme kinetics revealed that the optimum pH of the enzyme L-asparaginase was 8.0, optimum temperature was found to be 37^{0}C and optimum time was 15 minutes. The Km and V-max values of Spinacia oleracea was found to be 5 and 2.5 respectively by means of the double-reciprocal Lineweaver-Burk plot.

Keywords: L-asparaginase, *Spinacea oleracea*, UV-Visible Spectrophotometer, Enzyme activity and kinetics.

Introduction

L-asperaginase is a critical therapeutic enzyme that accounts for approximately 40% of the total enzyme sales[1]. The enzyme L-asparaginase is found to be responsible for the antitumor activity of guinea pig serum[2]. It is identified as an effective antitumor agent in human clinical trials and is now recognized as one of the important component of antitumor therapy. Currently this enzyme is used as a drug to treat acute lymphoblastic leukemia (ALL).

L-asparaginases derived from microbial sources especially *Escherichia coli* and *Erwinia chrysanthemi* are found to have anti-cancer activity. *E.coli* has thereby become the main source of L-asparaginase due to the fact that it is easy to produce in bulk. L-Asparaginase produced by *Erwinia chrysanthemi* is used as a drug under the trade name *Erwinase and E.coli* asparaginase is marketed under the brand name *Elspar* for the treatment of acute lymphoblastic leukemia (ALL). This enzyme can be given as an Intramuscular, Subcutaneous, or intravenous injection without fear of tissue irritation[3].

L-asparaginase (E. C. 3.5.1.1) is an enzyme that converts L-asparagine into L-aspartate and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate[4]. Asparagine is a nonessential amino acid produced in the human body by the enzyme asparagine synthetase Asparagine is required by the cells for the synthesis of protein. This amino acid is required in bulk by the lymphatic tumor cells for their rapid growth and multiplication. Hence these tumor cells also rely on the asparagine from the dietary supplement to meet their asparagine demand. When the dietary supplement of asparagine is cut off by the enzyme L-asparaginase it results in the death of the tumor cells. L-asparaginase is therefore a prominent enzyme that is used to treat Leukemia[6].

Few plants with significant amount of L-asparaginase have been reported. *Capsicum annum* and *Tamarindus indica* contain certain amount of L-asparaginase[7]. *Withania somnifera* is another source of this enzyme with high specific activity[8]. L-asparaginase has also been reported to occur in the maturing seeds of Lupinus albus and it has been detected in the plant fraction of Lupinus angustifolius nodules prior to the onset of N_2 fixation[9].

In the present investigation L-asparaginase was extracted from *Spinach oleracea*, an edible flowering plant of the family Amaranthaceae. Spinach is enriched with vitamin C, vitamin K, folic acid, calcium, iron, fiber and carotenoids[10]. It has dietary fiber which is essential for good digestion, treat constipation and maintain low blood sugar. Spinach contains flavanoids which is known as anti-cancer phytonutrient that slows down the cell division process of human stomach and skin cancer cells. It has a protective effect against aggressive prostate cancer[11]. Plant leaves were used to extract L-asparaginase enzyme for the present piece of work.

Materials and Methods

Extraction of crude enzyme: 1g of leaf sample is weighed, washed under tap water then rinsed with distilled water. Leaves are ground in a chilled mortar and pestle with two volumes of 0.1M phosphate buffer of pH7.5 at 3°C containing 5mM DTT and 1mM EDTA. Homoginization is carried out in chilled condition by placing ice bags below the mortar. The homogenate is passed through cheese cloth, centrifuged at

12,000 rpm for 30 minutes at 2-3°C. The supernatant serves as crude enzyme extract.

Estimation of total protein by Lowry's method: Working solution (200µg/ml) in aliquots of 0.2, 0.4, 0.6, 0.8 and 1mL is pippeted out into each test tube. The volume is made upto 1mL in each tube with 0.1N NaOH. 5mL of copper reagent is added to each tube. The tubes are incubated at 37°C for 10 minutes.0.8mL of F-C reagent is added and the tubes are incubated for 30 minutes at 37°C.Absorbance is read at 660nm.A graph is plot with the concentration of protein on X-axis and absorbance on Y-axis.

Standard graph of Ammonium sulphate by Nessler's Method: Ammonium sulphate working solution (50µg/ml) in aliquotes of 0.2, 0.4, 0.6, 0.8 and 1mL is pipetted out into each test tubes. The volume is made upto 3mL in each tube with distilled water.1mL of Nessler's reagent is added to each tube. Absorbance is read immediately at 460 nm using UV – spectrophotometer.A graph is plot with concentration of ammonium sulphate on X- axis and absorbance on Y-axis.

Enzyme assay of L-asparaginase in *Spinacia oleracea*: Four clean and dry test tubes are taken. The first tube is marked as "Blank", "Test 1 and 2" and a "control".0.5mL of substrate is added to each test tube.0.5mL of 50mM potassium phosphate buffer of pH 8.0 was added to each tube.0.25mL of 20%TCA was added only to the control tube.0.5mL of crude enzyme extract(***Spinacia oleracea***) was added to all the tubes except blank. The tubes were incubated for 15minutes at 37°C. 0.25mL of 20%TCA is added to all tubes except control. Tubes are centrifuged at 8000rpm for 5minutes.The supernatant is collected and the pellet is discarded.

To the supernatant 3mL of distilled water is added followed by 1mL of Nesseler's reagent.

Absorbance is read at 460nm using UV spectrophotometer.By using ammonium sulphate standard graph, the amount of ammonia liberated is determined.

Enzyme Kinetics: pH Kinetics:10 clean and dry test tubes were taken and labeled as pH 7.0- pH 9.0.0.5mL of L-asparaginase substrate was added to all tubes.0.5mL of 50mM potassium phosphate buffer was added to all tubes.The tubes has different pH 7,7.5,8.0,8.5, 9.0 and blank was maintained at pH8.0.25mL of 20% TCA was added to control tubes.0.5mL of crude enzyme extract (***Spinacia oleracea***)was added to all the tubes. The tubes were incubated at 37°C for 15minutes and then the reaction was stopped by adding 0.25mL of 20%TCA to the test tubes.The tubes were centrifuged at 8000rpm for 5minutes, the supernatant solution was collected.To the supernatant 3mL of distilled water and 1mL of Nessler's reagent was added. Absorbance read at 460nm using UV spectrophotometer. A graph was plotted with different pH values along x-axis and absorbance on y-axis.

Temparature Kinetics: Nine clean and dry test tubes were taken. The first tube was marked as 'blank', 'test' and 'control'. The tubes were taken which indicated various temperature 4, 27, 37,100.0.5mL of L-asparaginase substrate was added to all the test tubes.0.5mL of 50mM potassium phosphate buffer of pH 8 was added to each tube.0.25mL of 20% TCA was added to control tubes.0.5mLof crude enzyme extract (***Spinacia oleracea)*** was added to each tube.

The tubes were incubated for 15 minutes in their respective temperatures.0.25mL of 20% TCA was added to the tubes marked as 'Test'. The tubes were centrifuged at 8000rpm for 5minutes, the supernatant was collected. To the supernatant 3mL of distilled water and 1mL of nesseler's reagent was added.Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted with the temperature on X axis and absorbance on Y axis.

Time Kinetics:15 clean and dry test tubes were taken. The first tube was marked as 'blank', 'test' and 'control'.

0.5mL of L-asparaginase substrate was added to all the test tubes.0.5mL of 50mM potassium phosphate buffer of pH 8 to each tube.0.25mL of 20% TCA was added to control tubes.0.5mLof crude enzyme extract ***(Spinacia oleracea)*** was added to each tube.The tubes were incubated at 37°C for respective time 0,5,10,15,20,25, 30 minutes.

0.25mL of 20% TCA was added to the tubes marked as 'Test'. The tubes were centrifuged at 8000rpm *for* 5minutes, the supernatant was collected. To the supernatant 3mL of distilled water and 1mL of nesseler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer. A graph was plotted with the time on X axis and absorbance on Y axis.

Km and Vmax: Pipetted out different aliquots of Lasparaginase substrate (0.1-0.5 mL) in to different test tubes. The volume was made up to 0.5mLwith 50mM potassium phosphate buffer of pH 8.0.0.5mL of crude enzyme (***Spinacia oleracea***) was added to all the tubes. The tubes were incubated at 37°C for 15 minutes. 0.25mL of 20% TCA was added to all tubes. Tubes were centrifuged at 8000 rpm fo 5 minutes. Supernatant was collected. To the supernatant, 3mL of distilled water and 1mL of Nesseler's reagent was added. Absorbance was read at 460nm using UV spectrophotometer.

Results and Discussion

The leaf extract of spinacea oleracea was used as crude enzyme source and enzyme assay and kinetic parameters were carried out. The presence of the L-asparaginase enzyme in the leaves of spinacea was confirmed by UV-Visible spectroscopy at460nm. The characterization of L-asparaginase enzyme showed that there is considerable resemblance to the optimum conditions of this enzyme derived from microbial and plant sources.

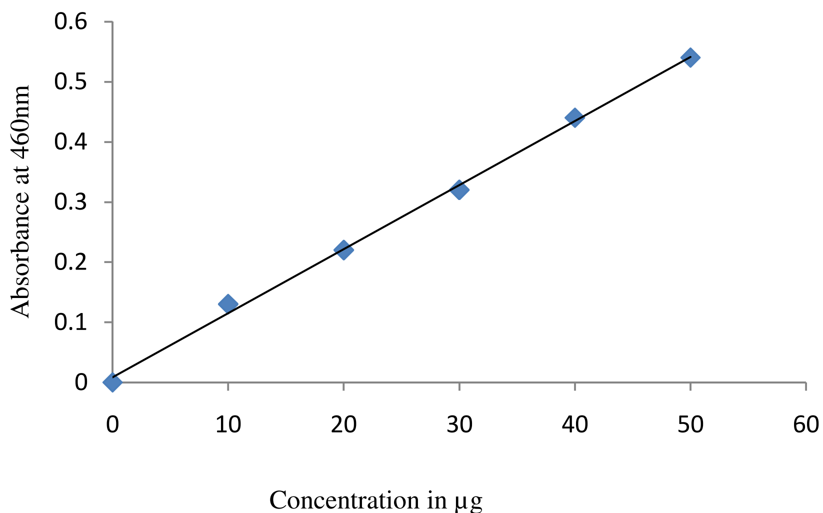

Figure-1
Ammonium sulphate standard graph to calculate the enzyme activity

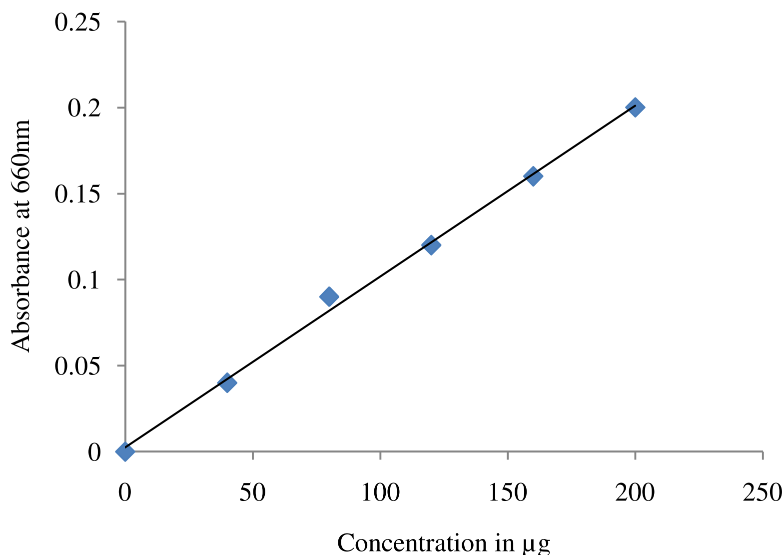

Figure-2
Lowry's standard graph to calculate the specific activity:

Table-1
Enzyme activity

Sl.no	Plant description	Absorbance at 460nm	Enzyme activity: IU
1	*Spinacia oleracea*	1.185	1.926

Table-2
Specific activity

Sl.no	Plant description	Absorbance at 460nm	Specification(µmoles/mg/min)
1	*Spinacia oleracea*	0.35	6.878

Enzyme Kinetics: pH kinetics: *Spinacia oleracea*

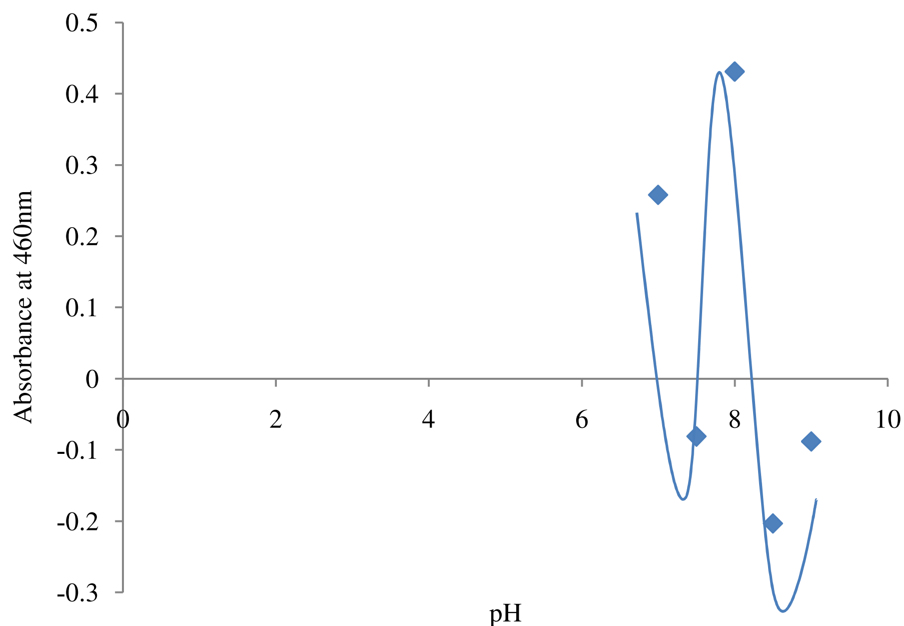

Figure-3
The optimum pH of L-asparaginase enzyme in *Spinacia oleracea* was found to be pH 8.

Temperature kinetics: *Spinacia oleracea*

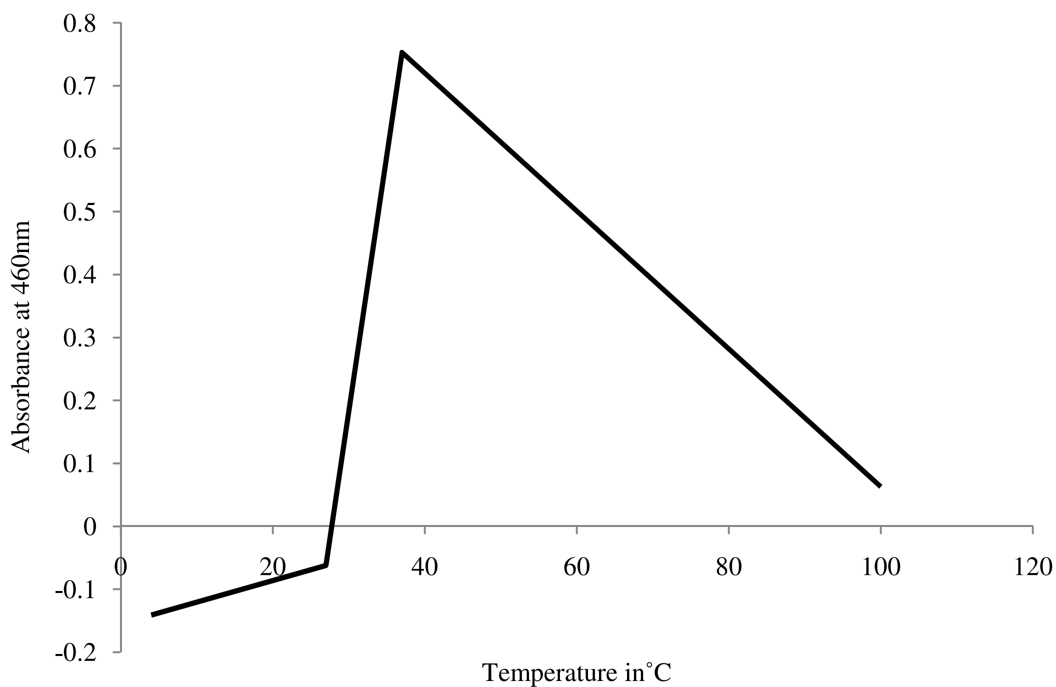

Figure-4
The optimum temperature of L-asparaginase enzyme in *Spinacia oleracea* was found to be 37°C.

Time kinetics: *Spinacia oleracea*

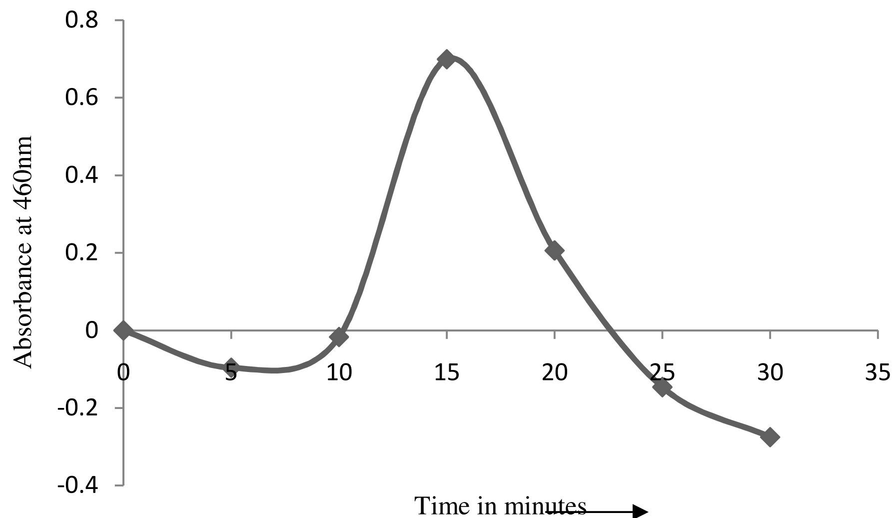

Figure-5
The optimum time interval of L-asparaginase enzyme in *Spinacia oleracea* was found to be 15 minutes

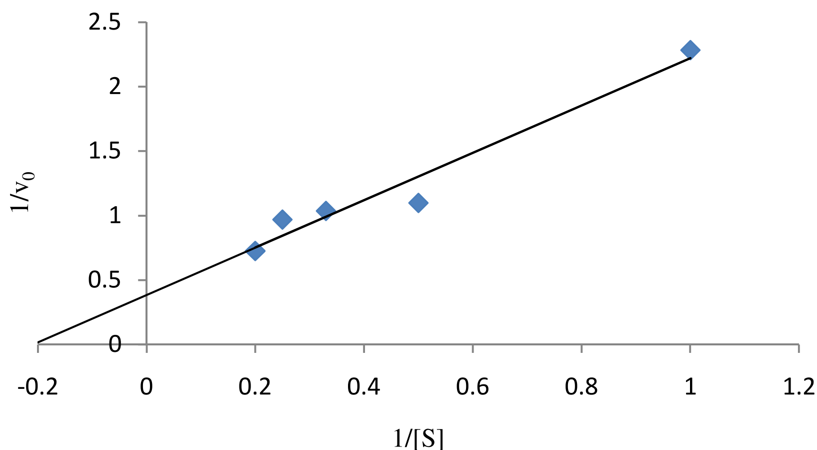

Figure-6
Km and Vmax

Table-3
Km and Vmax- *Spinacia oleracea*

Sl.no	[S] (in mg)	1/[S]	Vo	1/Vo
1	1	1	0.438	2.283
2	2	0.5	0.910	1.098
3	3	0. 33	0.965	1.036
4	4	0.25	1.032	0.968
5	5	0.2	1. 379	0.725

Km of Lasparaginase enzyme in ***Spinacia oleracea*** was found to be 5. Vmax of L asparaginase enzyme in ***Spinacia oleracea*** was found to be 2.5µmoles/ml/min.

Discussion: The plant Spinach oleracea was screened for L-asparaginase enzyme and was found to be present, with enzyme activity of 1.962IU and specific activity 6.878 µmoles/mg/min. The enzyme kinetics revealed that the optimum conditions are similar to that of the L-asparaginase isolated so far. pH of the enzyme L-asparaginase was found to be 8.0, optimum temperature was 37^0C and optimum time was found to be 15 minutes.Km and Vmax were determined by using different concentrations of L-asparagine as substrate. The Km and Vmax values of ***Spinacia oleracea*** was found to be 5 and 2.5 µmoles/ml/min respectively by means of the double-reciprocal Lineweaver-Burk plot.

Conclusion

Spinacia oleracea was found to be a good source of L-asparaginase, which showed similar kinetic parameters to the L-asparaginase reported till date. Hence further purification and characterization of this enzyme from *Spinacia oleracea* will count this plant as a medicinal herb to fight human blood cancer.

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