ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

CHARACTERIZATION OF L-ASPARAGINASE FROM *CAPSICUM ANNUM* L.

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Manuscript Info

Manuscript History:

Received: 14 March 2016
Final Accepted: 19 April 2016
Published Online: May 2016

Key words:
L-asparaginase, Capsicum annum L., Aspargine, Nesselerization, Enzyme Assay, Molecular weight.

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Abstract

L-asparagine is the most abundant metabolite for storage and transport of nitrogen that is utilized in protein biosynthesis in plants. L-asparaginase catalyzes the deamination of L-asparagine to aspartic acid and ammonia. The present study is aimed to characterize L-asparaginase from *Capsicum annum* L.Crude L-asparaginase was partially purified by salting out using 20% to 80% of ammonium sulphate. The activity of L-asparaginase was measured in the aqueous extract of fruits by Nesselerization method. The specific enzyme activity after 80% saturation was 4841.78 IU/mg. Optimum incubation period for L-asparaginase was found 60 min. The enzyme showed stability at alkaline pH and has pH 8 as optimum pH. L-asparaginase have maximum activity at 37^{o}C. Highest activity of L-asparaginase was at 6mM substrate concentration. Kinetic parameter study revealed high affinity of enzyme towards L-aspargine with high velocity. Partially purified enzyme have molecular weight of 25.41 kDa estimated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Introduction:-

Lang was the first person who found Asparaginase activity in Beef tissues which confirmed by Furth and Friedmann [3]. L-asparaginase (L-aspargine amino hydrolase EC3.5.1.1) is enzyme catalyzing deamination of L-aspargine into aspartic acid and ammonia [4]. According to amino acid sequences and biochemical properties L-asparginase have two broad categories Bacterial and Plant Asparaginase [4]. In plants Asparaginase is predominantly found in developing leaves and seeds. *Capsicum annum, Pisum sativum, Viga unguicalata, Withania somnifera, Tamarindus indica* are some of the examples of plant L-asparaginase producers [5]. L-asparaginase is a therapeutically important enzyme in combination with the other drugs in the treatment of various types of blood cancer such as acute lymphocytic leukemia, Hodgkin disease, acute myelocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma surrounding tissue. L-asparaginase has also been used for making a diagnostic biosensor as the amount of ammonia produced by the action of the enzyme directly correlates to the level of L-aspargine in patients' blood. L-asparaginase also play important role in food processing as acrylamide formation in fried and baked foods by the reaction of asparagine with reducing sugar which shows neurotoxicity in humans [6].

Therapeutically important L-asparaginase is isolated from *Erwinia chrysanthemi* and *E.coli*. Drug from mentioned sources are showing allergic reactions in 50% cancer patients and cause liver disfunction, pancreatic, leucopenia, neurological seizures and coagulation abnormalities leading to intracranial thrombosis or hemorrhage due to its low substrate specificity and high glutaminase activity [7],[8]. Thus there is a need to have L-asparaginase with no side effects.The objective of present study is characterization of L-asparaginase from plant *Capsicum annum* L.

Materials and methods:-

All chemicals and reagents are of analytical grade purchased from Merk, HiMedia and s.d. fine chemicals.

Collection:-

The fresh 'Chilies' of *Capsicum annum* L. were collected from 'Phulambri' Aurangabad, Farm site. Authentication of plant was done from Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (Accession no. 0592).

Crude Extraction preparation:-

Collected Chilies sample washed thoroughly with tap water followed by distilled water to remove surface dust and extraneous material. 500 gm fresh green chilies having length 5 to 6 cm long and 1 to 2 cm broad were collected and homogenized by using 1500 ml KCL having molarity 0.15 M. The homogenized sample was filtered with the help of muslin cloth and Centrifuged at 4^0C at 10,000rpm for 15min and the supernatant was collected as source of enzyme. The obtained crude extract was analyzed for enzyme activity. The crude extract was used for partial purification. [4], [9], [10].

Enzyme Assay:-

In enzyme assay 0.9 ml of asparagine (4 mM), 0.1 ml Tris-HCl buffer [50 mM, pH 8.0] and 0.1ml enzyme was incubated for 1 hr at 37^oC.The reaction was stopped by adding 0.1 ml of Trichloro Acetic Acid (TCA).The given mixture was centrifuged at 10,000 rpm for 5 min and the liberated ammonia was estimated by adding 100µl of Nessleres reagent . After incubation of 10 min absorbance at 425 nm was measured [11]. Protein concentration was determined by Follin-Lowry method.

One unit of L-asparaginase activity is defined as the amount of the enzyme that liberates 1 µmol of ammonia per minute at 37^0C [4].

Partial Purification:-

Partial purification was accomplished by ammonium sulphate precipitation (20% to 80%). Precipitate was dissolved in phosphate buffer (pH7.4).The sample was dialyzed for overnight at 4^0C against phosphate buffer. Dialyzed sample concentrated on sucrose and stored at 4^0C.The dialyzed sample proceed for detection of protein concentration and enzyme activity. Partially purified enzyme was used for optimization of incubation period, pH, temperature, substrate concentration and calculation of kinetic parameter and Molecular weight determination [12].

Characterization of L- asparaginase activity:-

1. Optimum Incubation period: The optimum incubation period for enzyme activity was determined by incubating the assay mixture at different time periods i.e., (10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min, 90 min) and enzyme activity was detected [11].
2. Optimization for pH: The enzyme was incubated at 37^0C with citrate buffer (for pH 3.0, 4.0, 5.0) phosphate buffer (for pH 6.0, 7.0, 8.0) and Tris-HCl buffer (for pH 9.0,10.0,11.0) under assay conditions and enzyme activity was determined [13].
3. Temperature tolerance: The optimum temperature for the enzyme activity was determined by incubating the assay mixture at different temperatures i.e., (30^0C, 37^0C, 50^0C, 60^0C and 70^0C, 80^0C) and enzyme activity was analyzed [13].
4. Effect of Substrate Concentration: The effect of substrate concentration for enzyme activity was determined by incubating the assay mixture at different substrate concentration (2mM, 4mM, 6mM, 8mM, 10mM, 12mM, 14mM, 16mM, 18mM, 20mM) and enzyme activity was detected.
5. Kinetic parameter: The Km and Vmax values of L – asparaginase were calculated from the reciprocal plots of substrate concentration versus reaction velocity or enzyme activity (i.e., L.B plot) [13].

Molecular weight determination by SDS-PAGE:-

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed for molecular weight determination.Stacking gel of pH 6.8 with 4% of acrylamide-bis acrylamide and separating gel with pH 8.3 and 12% acrylamid-bis acrylamide was utilized [11], [13].

Result and discussions:-

Accession no:- Accession no. of *Capsicum annum* L. is 0592.

Partial purification by Ammonium sulphate precipitation:-

Table I:- Comparison of crude extract and partially purified enzyme.

No.	Name	Protein concentration (mg)	Enzyme activity (uM/ml/min)	Specific Activity (IU/mg)	Fold Purification	Yield (%)
1	Crude extract	0.060	252.32	4205	-	100
2	Ammonium sulphate precipitation 80%	0.028	135.57	4841.78	1.15	53.72

The potassium chloride extraction was followed by partial purification of L- asparaginase by ammonium sulphate precipitation yielded 53.72% which less than *Penicillium* spp. 97.181% [14] and that of marine actinomycetes 65.83% [15].The purity of enzyme 1.15was found to be near *Penicillium* spp. 1.115 [14] and marine actinomycetes 1.09 [15], [9].

Characterization of L- asparaginase activity:-

1. Optimum Incubation period: The optimum incubation period for enzyme activity was determined by incubating the assay mixture at different time period (10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min,90 min) and enzyme activity was detected. The maximum Enzyme activity was detected at 60 minute.

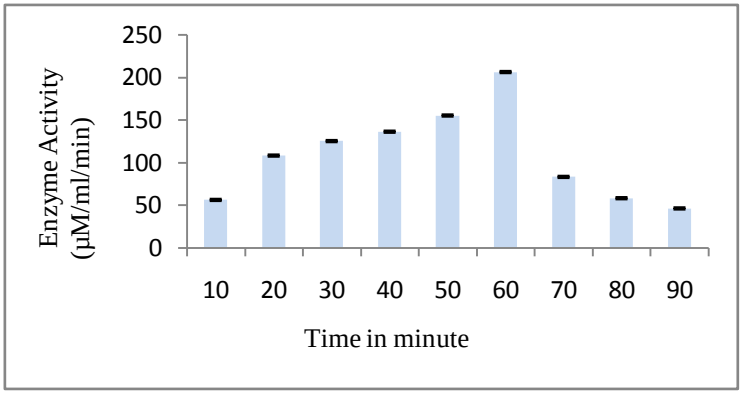

Fig. 1:- Optimum Incubation period.

2. Optimization for pH: The enzyme activity of L – asparaginase was maximum at pH 8.0. The enzyme is active at pH 7-pH 9.

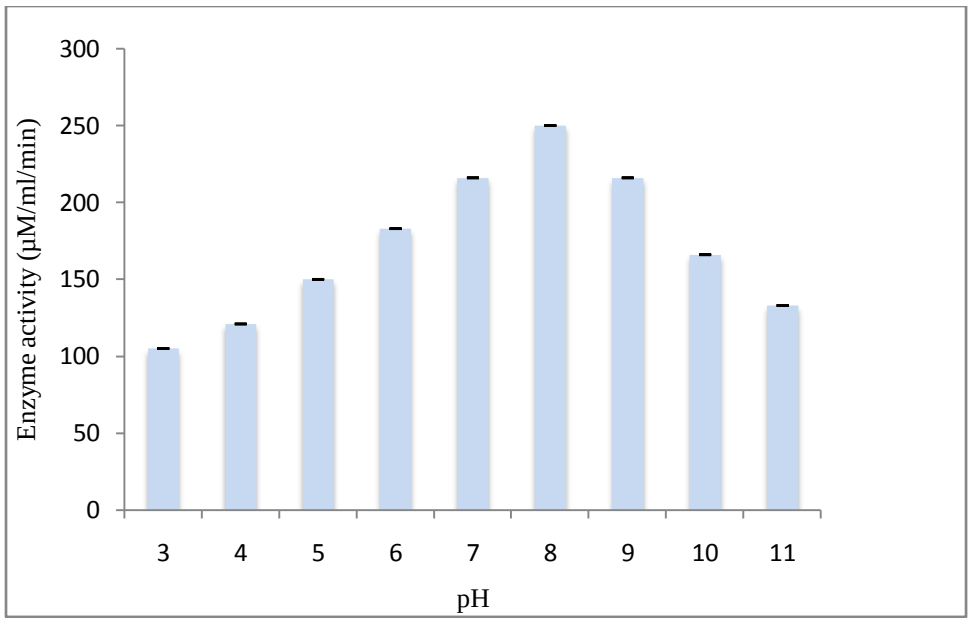

Fig.2:- Optimization for pH.

The partially purified L- asparaginase from *Capsicum annum* L. was functionally stable and active over wide range of pH and highest activity at pH 8.0 which is less than that of L- asparaginase isolated from *E.coli* and *Erwinia* [11]. The optima is greater than *Streptomyces* spp. which is 7.0 – 7.5 [12].

3. Temperature tolerance: It is evident from fig.3 that gradual increase in enzyme activity of L-asparaginase upto 37^0 C and then decrease. The enzyme activity was gradually declined beyond 40^0C.

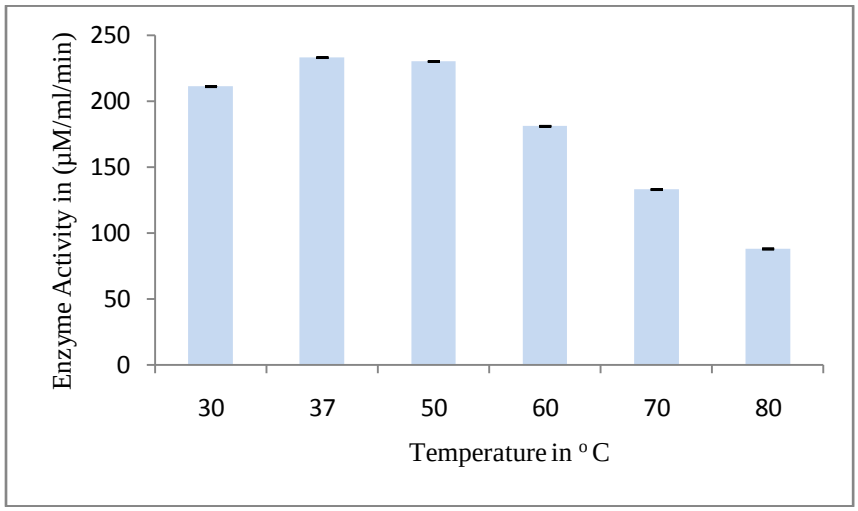

Fig. 3:- Temperature tolerance.

Maximum activity of L- asparaginase in present study was found at 37^0 C, declined as temperature increase which is same to *E.coli* [9] and *Erwinia*.[11]

4. Effect of Substrate concentration: The effect of substrate concentration for enzyme activity was determined by incubating the assay mixture at different substrate concentration (2 mM, 4mM, 6mM, 8mM, 10mM, 12mM,

14mM, 16mM, 18mM, 20mM) and enzyme activity was detected. Optimum Enzyme activity was detected at 6mM substrate concentration after that it was found drop in enzyme activity for substrate concentration 8mM and then it was steady. It may be due to L-asparaginase is partially purified.

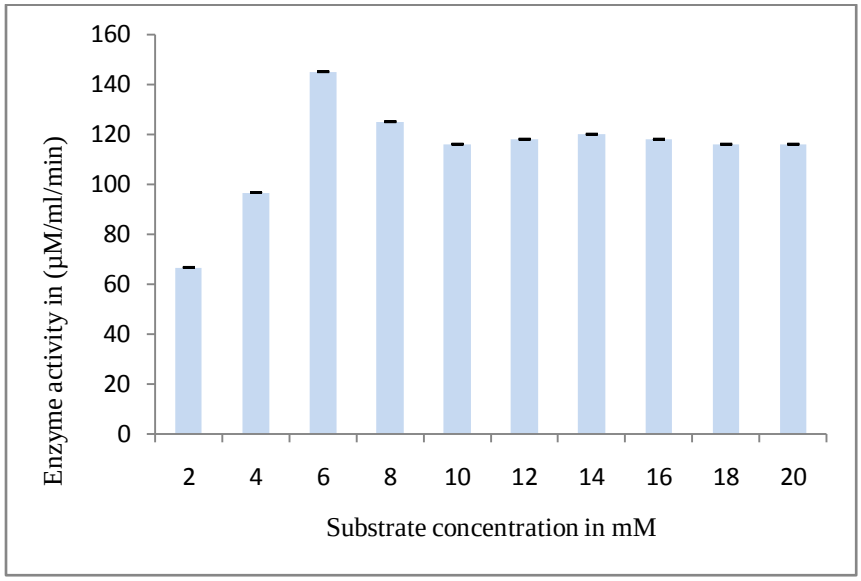

Fig.4:- Effect of Substrate concentration.

5. Kinetic parameters: The Km and Vmax values of L-asparaginase were calculated from the reciprocal plots of substrate concentration versus reaction velocity.

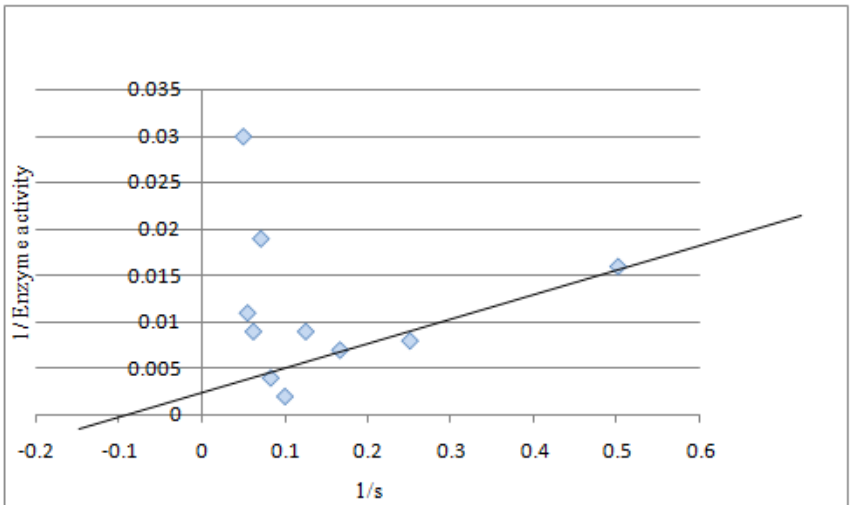

Fig.5:- Kinetic parameters.

Line weaver – Burk double reciprocal plot of partially purified L-asparaginase shows Km value 12.5 mM which is maximum as compare to purified L-asparaginase of *E.coli* (0.0125mM)[9] and *Erwinia aroideae* (0.098mM), *Withania somnifera* (0.075 mM) [13] and *Capsicum annum* (3.3mM) [9]. Line weaver – Burk double reciprocal plot shows that Vmax of *Streptomyces griseoluteus* (9.60 IU/ml) [12] and which is lower as compared to Vmax of *Capsicum annum* L. 400 IU/ml in present study, lower than *Withania somnifera* 526.31U/min.[13].

Determination of molecular weight: By control with known molecular weight (L-asparaginase), it was determined that the molecular weight of L-asparaginase from *Capsicum annum* L. was found to be 25.41 kDa as shown in Figure 6.

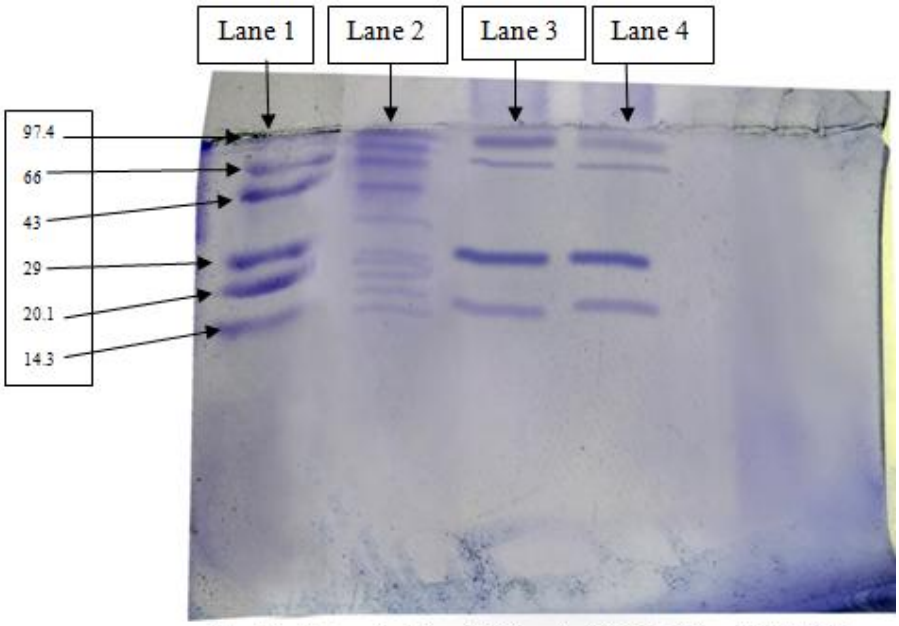

Fig. 6:- Determination of Molecular Weight Using SDS-PAGE

Lane 1: Protein Marker
Lane 2: Crude Extract
Lane 3: Partially purified L- asparaginase
Lane 4: Partially purified L- asparaginase

In present study molecular weight determination by SDS-PAGE of partially purified L-asparaginase with positive control, found to be 25.41 kDa which is nearer to *Withania somnifera* (36 kDa) [13]. The molecular weight 120000Da found on polyacylamide gel electrophoresis in *Capsicum annum* L.[9].

Conclusion:-

In this study, L-asparaginase enzyme extracted from *Capsicum annum* L. after partial purification with ammonium sulphate (80%) completes its reaction in 60min incubation with maximum enzyme activity at 37^0C at pH 8.0. The L-asparaginase is stable at alkaline pH as well as temperature tolerant. The enzyme got saturated at 6mM substrate concentration.Molecular weight pattern observed on SDS-PAGE which indicates band of 25.41 kDa. Kinetic parameters also determine (Km 12.5 mM and Vmax 400 IU/ml).

Acknowledgment:-

The authors wish to thank Dr. P.V. Ashtekar, Principal, Shivchhatrapati College, Aurangabad and Mrs. Savita Kate, Head of the Department of Biotechnology, Shivchhatrapati College, Aurangabad for encouragements.

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