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Comparative study of L-asparaginase from different cytotypes of *Withania somnifera* (L.) Dunal and its purification

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ABSTRACT

Present scenario has focussed that L- asparaginase has been found to be best anti leukemic agent. It has been characterised based on the assay principle hydrolyzing L-asparagine into L-aspartic acid and ammonia. Withania somnifera (L.) Dunal (Ashwagandha); Tradtional ayurvedic medicinal plant, used in treatment of various diseases, found to have good source of enzyme L-asparaginase. In present study, different cytotypes of W. somnifera were compared for ploidy level and L-asparaginase activity. L-asparaginase was extracted from diploid and tetraploid Withania somnifera plants and tetraploid was found to have three times higher activity. Enzyme was purified using ammonium sulphate precipitation, Ion exchange and gel permeation chromatography. Purified L-asparaginase revealed molecular weight 75 kDa, Km value 1.5 mM and Vmax 526.31 µM/min. Purified enzyme has 61.22 % yield with 13.87 fold increase in specific activity.

Key words: *Withania somnifera* (L.) Dunal, Cytotypes, L-asparaginase, Acute Lymphoblastic Leukaemia (ALL), purification.

INTRODUCTION

L- asparaginase is an anti –leukemic enzyme hydrolyses asparagine into aspartic acid. There are a wide range of sources of L-asparginase i.e. bacteria, algae, fungi, plants etc. Among plants, Ashwagandha (*Withania somnifera* (L.) Dunal) is considered as the good source [1] which is also known as Indian Ginseng, one of the most widely used herbs in medical system. This plant has anti-inflammatory [2], anticancer [3], antistress, immunomodulatory [4] and cardiovascular activities [5]. Thats way Ashwaganda is admired in ayurveda from ancient time. The root extract of *W. somnifera* has also been used as a dietary supplement in the United States. It is more commonly found in Africa and Indian subcontinents. In India, it grows in dry parts of tropical and subtropical regions. Out of its three cytotypes, diploids and tetraploids are frequently available [6] but there is only one report of hexaploids [7].

In present study, we compared these cytotypes in terms of L-asparaginase activity. As a therapeutic agent, L-asparaginase is used for the treatment of acute lymphocytic leukemia (ALL). In ALL, leukemic cells do not express adequate quantity of asparagine synthetase enzyme and are dependent on the extracellular asparagine. During the treatment with Asparaginase, serum concentration of asparagine decreases due to hydrolysis of asparagine into aspartic acid and ammonia. As the cells get starved of asparagine, protein synthesis is reduced in cells that are exposed to Asparaginase. Thus, the cells are killed due to insufficient amount of asparagine in the circulating pool. It is also used for the treatment of pancreatic carcinoma and bovine lymphomosarcoma [8]. Asparaginase obtained from *E.coli*, is used as a drug under the brand name Elspar for the treatment of acute lymphoblastic leukaemia (ALL) and can be given as an intramuscular, subcutaneous, or intravenous injection without fear of tissue

irritation. Asparaginase marketed under the brand names Acrylaway and PreventASe, are used to reduce the formation of acrylamide in starchy food products such as snacks and biscuits [9].

MATERIALS AND METHODS

All the chemicals used were of analytical grade, commercially available from Himedia, Sigma, Merck etc. Otherwise mentioned.

Cytological studies [6]
Plants, for cytological studies, were collected from different places of Punjab state and adjoining areas. Sample Plant was submitted in Herbarium of Department of Botany, Punjabi University, Patiala and got the accession No. PUN 57617. Floral buds of suitable size were fixed in Camoy's fixative for 24 hrs and preserved in 70% alcohol at 4^{o} C. For meiotic studies, anthers were squashed in 1% acetocarmine and slides were carefully examined for chromosome counts in each sample.

Extraction of enzyme [10]
L-asparaginase was extracted from green fruits of different cytotypes of *Withania somnifera*. For enzyme extraction fruits were washed with distilled water and crushed with sterilized chilled sea sand at 4^{o}C. The same amount of sodium borate buffer (pH 8.6) was added to the finely crushed mixture and then centrifuged at 8000 rpm for 10 min at 4^{o}C. The supernatant was collected and used for measuring enzyme activity.

L-asparginase assay [11]
1.7 ml of 0.01M L-asparagine (prepared in 0.05M Tris-HCl) and 0.2 ml 0.05M Tris-HCl were added to a test tube. To this, 20µl enzyme (diluted to 1ml with Na borate buffer) was added and incubated at 37 ^{0}C for exactly 10 minutes and reaction was stopped by adding 0.1ml of 1.5 M Tri Chloroacetic Acid. Reaction mixture was clarified by centrifugation and 2.5 ml clear supernatant was mixed to equal volume of de-ionized water. To this, 0.5ml Nessler's reagent was added and incubated at room temperature for 10 minutes. Absorbance was taken at 480 nm and amount of ammonia released was determined using an ammonium chloride standard curve.

Purification of L-asparaginase
L-asparaginase was purified by the following steps according to Scope [12].

Ammonium sulphate fractionation
Ammonium sulphate was added to 20 to 100% saturation. The mixture was left for 12 h at 4^{o}C, followed by centrifugation at 8000 rpm for 20 min at 4^{o}C. The precipitate from each saturation fraction was dissolved in 0.01M Na Borate buffer (pH 8.6) and dialyzed overnight against the same buffer at 4^{o}C.

Gel filtration
The ammonium sulphate fraction with maximum specific activity was applied to Sephadex G-75 column, preequilibrated with 0.01M Sodium Borate buffer (pH 8.5). Dimension of column was 1.2 X 9.2 cm. Sample was eluted at a constant flow rate of 1 ml/min and fractions of 2 ml were collected. The fractions were assayed for protein concentrations and enzyme activity. Fractions having higher specific activity were pooled and used for ion-exchange chromatography.

Ion-exchange chromatography
Anion-exchange chromatography was carried using Q-sepharose. The **c**olumn was preequilibrated with 0.01M Na Borate buffer (pH 8.6). Dimension of column 1.2 X 8.2 cm. Protein fractions were eluted by increasing salt gradient (NaCl,0.1M-0.6 M).

Electrophoresis
11% PAGE was performed as previously described [13] to check the purity and molecular weight of purified protein by using Biorad assembly. Proteins were visualized by silver staining [14].

Kinetic Properties
Km and Vmax were evaluated by Lineweaver-Burk double reciprocal plot. Effect of different pH (6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5) and temperature (25^{o}C, 30^{o}C, 35^{o}C, 37^{o}C, 40^{o}C, 45^{o}C and 50^{o}C) were also studied.

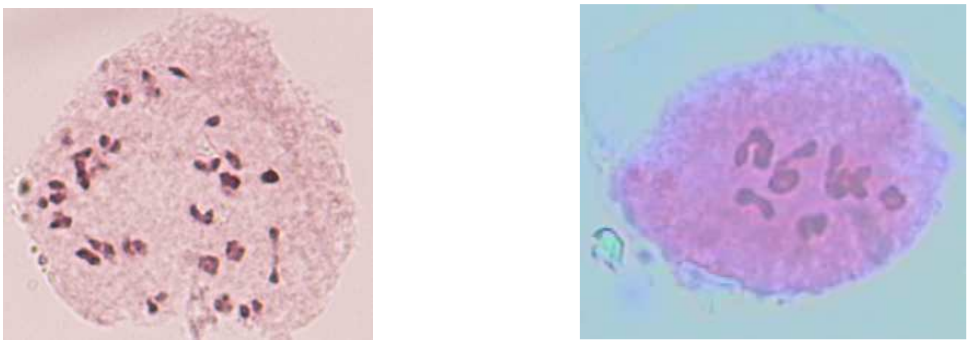
RESULTS AND DISCUSSION

Cytological study

Withania somnifera plants were collected from different places Punjab and adjoining areas as shown in table 1. Many studies have compared the cytotypes of *W. somnifera* but first time cytotypes were compared with respect to L-asparaginase activity in present study. Only two types of cytotypes were found i.e. diploid and tetraploid (figure 1.). Tetraploids showed three times increased L-asparaginase activity in comparison to diploids (Table 2). This may be simply due to the fact that tetraploid plant have extra sets of chromosomes than diploid therefore having more genes than diploid plants. Among the three stages of fruit (i.e. green→ young bud, red→ half ripened, dry → fully ripened), the green stage had maximum enzyme activity. Green stage has developing seeds and it has been documented that developing seeds require asparagine as nitrogen source [15,16,17]. Furthermore, metabolism of asparagine essentially require L-asparaginase. Protein extracts from tetraploid plants were used for enzyme purification.

Table 1: collection of *Withania somnifera* plants from different regions

location	No. of plants	Ploidy level	
		4X	2X
Mohali	8	8	0
Solan	7	4	3
Sunam (Sangrur)	5	5	4
Patiala	11	11	0



(a) **(b)**

Figure 1: (a) Metaphase-1 showing 24 bivalents i.e. tetraploid (4X), (b) Metaphase-1 showing 12 bivalents i.e. diploid (2X).

Table 2: Comparison cytotypes for L-asparaginase

Cytotype	Enzyme activity
Tetraploid (4X)	13 IU/g
Diploid (2X)	4 IU/ g

Purification

The results for purification of L- asparaginase are summarized in Table 3. The specific activity of the crude extract was 1.66 units/ mg protein. 40-60% saturation of ammonium sulphate fractionation showed 4.6 fold purification of enzyme with 78.88% recovery. L-asparaginase from other plants i.e. Green chilli [18], Pea [19] etc also showed maximum activity in same fraction. Activity was further increased by 8.2 fold after gel permeation chromatography using Sephadex G-75 column, though enzyme recovery was reduced to 64.11%. Maximum enzyme activity shown by 4^{th} and 5^{th} fraction of gel permeation (figure 2) and pooled out for ion exchange. The enzyme was further purified by strong anion exchange chromatography using Q- sepharose, enzyme yield was 61.22 % with 13.87 fold increase in specific activity. L-asparaginase was eluted with 0.5M NaCl solution (figure 3). Electrophoresis study reveals that single band in fourth lane shows the purified enzyme (Figure 4). Approximate molecular weight of purified enzyme was found to be 75 kDa, as determined by comparing it against protein marker ladder on gel. Oza *et al*., [1] reported the molecular weight of L-asparaginase as 72kDa which is comparable to present study. In one of their study, Chagas and Sodak purified the L-asparaginase from testa of immature pea seed and reported that its molecular weight was 69 kDa [19].

Table 3: Purification of L-asparaginase

Sample	Enzyme units	Protein (mg)	Specific activity	Fold	% recovery
Crude	340	205	1.66	------	100
40- 60%	268	35	7.65	4.6	78.88
Gel permeation	218	16	13.62	8.2	64.11
Ion Exchange	210	9.12	23.02	13.87	61.76

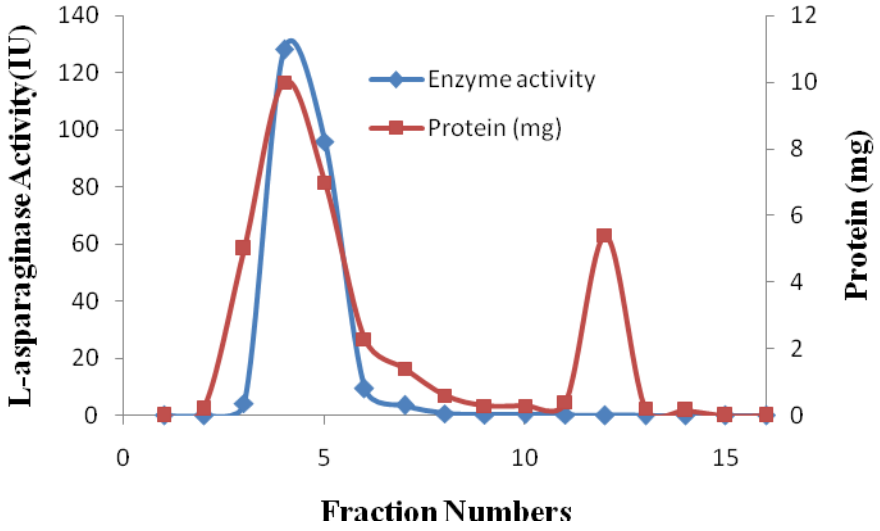


Figure 2: Sephadex G-75 chromatography of L-Asparaginase showing protein profile and enzyme activity profile.

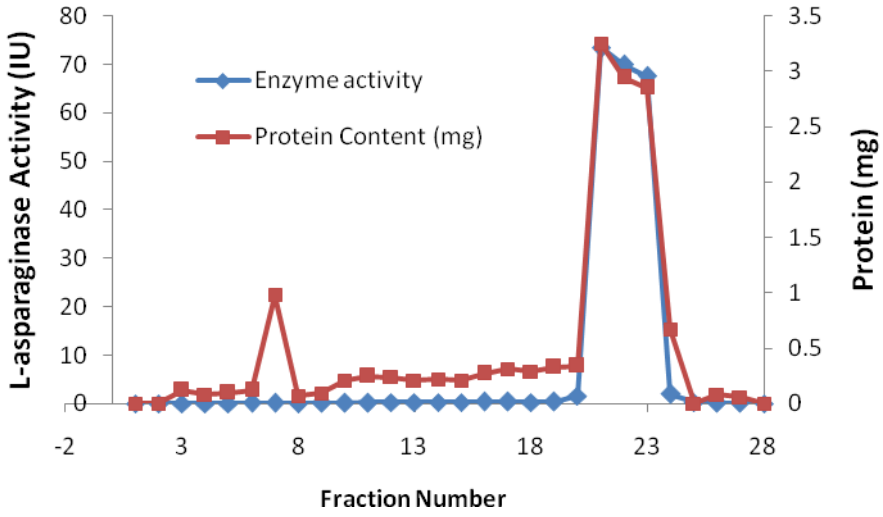


Figure 3: Q-sepharose ion exchange chromatography of L-Asparaginase showing specific activity profile.

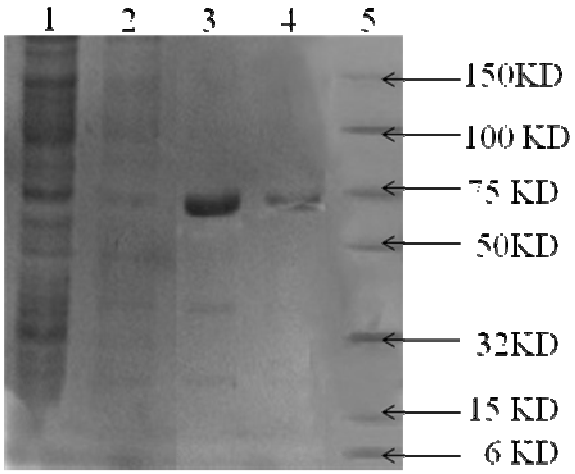


Figure 4: Shows the result of PAGE. In first lane crude enzyme, second lane has fraction of ammonium sulphate precipitation, third lane shows the fraction of gel filtration and fourth lane has a single band of fraction of Q-sepharose column. 5th lane has broad range protein marker.

Kinetic properties

Lineweaver-Burk double reciprocal plot shows that Km is 1.5 mM which is lower than L-asparaginase purified from green chilli (3.3mM) [18] and from Pea (2.4mM) [19] and the Vmax is 526.31 µM/min (figure 5). Km is low when its compared with Lineweaver-Burk double reciprocal plot of L-asparaginase by Oza *et al.*,[1] i.e. 20mM and Vmax is almost comparable (714.28 µM/min). Purified enzyme had maximum activity at pH 8.5. Most of the L-asparaginase from bacteria and plants have maximum activity at same pH (figure 6). Maximum enzyme activity was found at 37^{o}C (figure 7). The optimum temperature of L-asparaginase from *W. somnifera* is similar to all bacterial and plants L-asparaginases.

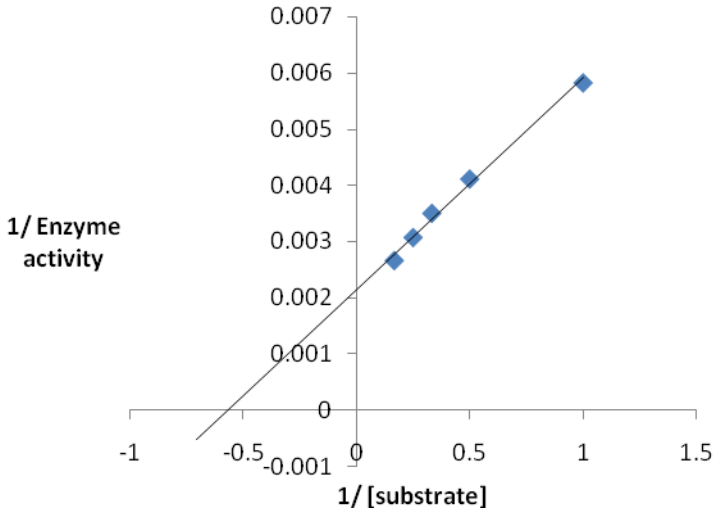


Figure 5: Lineweaver-Burk double reciprocal plot for L-asparaginase

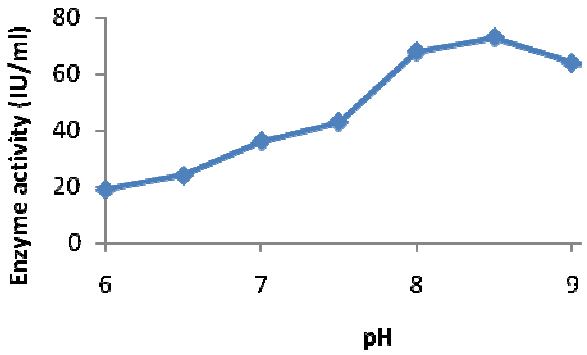


Figure 6: Effect of pH on enzyme activity

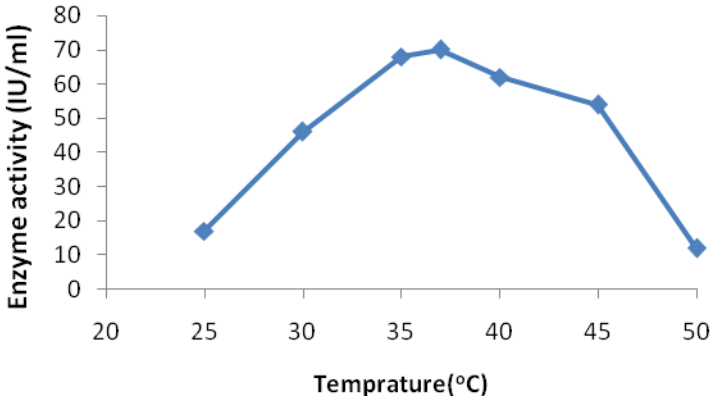


Figure 7: Effect of different temperature on enzyme activity.

CONCLUSION

Two cytotypes (Diploid and tetraploid) of *Withania somnifera* were screened for L-asparaginase with tetraploid found to have three times higher activity. Hence proceeded for further study. Purified enzyme has 61.22 % yield with 13.87 fold increase in specific activity. Purified L-asparaginase revealed molecular weight 75 kDa, km value 1.5 mM and Vmax 526.31 µM/min. As the purified enzyme has better kinetics this can be used as a biocomponent for the construction of asparagine biosensor. The purified enzyme will be better therapeutic agent than bacterial asparaginase since it has many side effects but *W. somnifera* is used in ayurveda without any complications.

REFERENCES

[1] VP Oza; SD Trivedi; PP Parmar; RB Subramanian. *J. Integr. Plant Biol.*, **2009**, *51*(2), 201-206.
[2] M Rasool; P Varalakshmi. *Vasc. Pharmacol*., **2006,** *44*, 406–410.
[3] R Mohan; HJ Hammers; PB Mohan; XH Zhan; CJ Herbstritt; A Ruiz. *Angiogenesis*, **2004,** *7*, 115–122.
[4] D Rai; G Bhatia; T Sen; G Palit. *J. Pharmacol. Sci.*, **2003**, *93*, 458–464.
[5] I Mohanty; DS Arya; A Dinda; KK Talwar; S Joshi; SK Gupta. *Basic Clin Pharmacol Toxicol*, **2004**, *94*,184–190.
[6] VK Singhal; P Kumar. *Comparative Cytogenetics*, **2008,** *2*(1), 85-92.
[7] SS Bir; Neelam. *J. Cytol. Genet.*, **1984**, *19*, 113-114.
[8] MA Mosterson; BL Hull, and ; LA Vollmer. *J. American Vet. Med. assoc.*, **1988**, *192*, 1301-1306.
[9] Food safety newsletter, **2008**, *315*(7/20). (www.FoodHACCP.com)
[10] DM Bollag; MD Rozycki; SJ Edelstein. In Protein Methods, 2nd ed, John Wiley & Sons, New York, 1996.
[11] A Meister. *Methods Enzymol.*, **1955**, *2*, 383.
[12] KR Scopes. In Protein purification: Principles and practice, 3rd ed, Springer -Verlag, New York,1994.
[13] UK Laemmli. *Nature*, **1970**, *227*, 680-685.
[14] B Oakley; D Kirsch; NR Morris. *Anal. Biochem.*, **1980,** *105*, 361-363.
[15] JF Thompson; JT Madison. *Ann. Bot*., **1977**, *41*, 29-39.
[16] PJ Lea; JS Hughes; BJ Miflin. *J. Exp. Bot*., **1979**, *30*, 529-37.
[17] KI Haga; L Sodek. *Ann. Bot*., **1987**, *9*, 597-601.

[18] M Bano; Sivaramakrisnan . *J. Biosciences*, **1980**, *2*, 291-297.
[19] EP Chagas; L Sodek. *Braz. arch. biol. technol.*, **2001**, *44*(3) 239-245.