Genetic characterization of L-asparaginase gene found in selected members of family Solanaceae

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Genetic characterization of L-asparaginase gene found in selected members of family Solanaceae

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Master of Philosophy

In

Plant Genetics and Genomics

By

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2014

DECLARATION

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other source of information has been used, they have been acknowledged.

USMAN ALI

CERTIFICATE

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TABLE OF CONTENTS

	Titles	Page No.
1	List of Tables	i
2	List of Figures	ii
3	Acknowledgements	iii
4	List of Abbreviations	v
5	Abstract	vii

Chapter 1	**Introduction**	**Page No. 1**
1.1	Review of Literature	03
1.2	Molecular structure	04
1.3	Mechanism of action	04
1.4	Metabolic pathways triggered by Asparaginase	05
1.5	Side effects of L asparaginase	07
1.6	Immunogenic complictaions/Resistance to the drug	09
1.7	Chemical modification of the enzyme	10
1.8	Sources of L-asparaginase	12
1.8.1	Bacterial L-asparaginase	13
1.8.2	Fungal L-asparaginase	15
1.8.3	Actinomycetes L-asparaginase	16
1.8.4	Algae L-asparaginase	17
1.8.5	Plant L-asparaginase	17
1.9	Aims and Objectives	20

Chapter 2	**Materials and Methods**	**Page No. 21**
2.1	Plant materials	21
2.2	Genomic DNA Extraction	21

2.3	Isolation of Total RNA	22
2.3.1	cDNA synthesis	22
2.4	Amplification of L asparaginase gene	23
2.4.1	Primer designing	23
2.4.2	Polymerase Chain Reaction	24
2.4.3	Agarose Gel Electrophoresis of PCR Products	24
2.4.4	Elutions of DNA Bands from Agarose Gel	24
2.4.5	Sequencing of the Gene	25
2.5.1	Analysis through Bioinformatics Tools	25
2.5.2	Translation of nucleotide sequence into amino acid sequence	25
2.5.3	Determination of PI/Mw value	25
2.5.4	Determination of Physiochemical Properties	25
2.5.5	Hydropathy Plot	26
2.5.6	Secondary structure of L-asparaginase	26
2.5.6.1	Protein Secondary structure map	26
2.5.7	Intrinsic disordered profile	26
2.5.8	Gene Ontology terms prediction	26
2.5.9	Alignment results	26

Chapter 3	**Results**	**Page No. 27**
3.1	Genomic DNA extraction	27
3.1.1	PCR amplification of L-asparaginase from Genomic DNA	28
3.2	Total RNA	29
3.2.1	cDNA synthesis	30
3.2.2	PCR amplification of L-asparaginase gene from cDNA	31

3.3.1	Analysis through Bioinformatics tools	32
3.3.1.1	Translation of nucleotide sequence into amino acid sequence	32
3.3.1.3	Physiochemical properties	32
3.3.1.4	Hydropathy plot	33
3.3.1.5	Secondary structure of L-asparaginase	34
3.3.1.6	Intrinsic Disordered Profile	36
3.3.1.7	Gene Ontology terms prediction	37
3.3.1.8	Amino acid Percentage	38
3.3.1.9	Alignment results	40

Chapter 4 **Discussion** **Page No.(42-44)**

Conclusion	45
Future Recommendations	46
References	47
Appendices	61

LIST OF TABLES

Table No.	Title	Page No.
1.1	Comparison of native and modified L asparaginase	11
1.2	Various Chemical modifications of L asparaginase	12
1.3	Biochemical properties of some bacterial Asparaginases	14
1.4	Biochemical properties of Fungi L- asparaginase	16
1.5	Biochemical properties of Plant L asparaginase	19
2.1	PCR Temperature profile for cDNA synthesis	23
2.2	L asparaginase primers designed for Genomic DNA and cDNA	23
3.1	Physiochemical properties	32
3.2	Biological functions	37
3.3	Molecular functions	38
3.4	Amino acid percentage	39
3.5	Alignment results	41

LIST OF FIGURES

Figure No.	Figure	Page No.
1.1	Asparagine degradation carried out by L asparaginase	05
1.2	Schematic diagram for metabolic pathways triggered by L asparaginase	07
3.1	Extracted DNA from immature fruits	27
3.2	Extracted DNA from leaf	27
3.3	PCR amplification of L asparaginase gene from Genomic DNA	28
3.4	Extracted RNA from immature fruits	29
3.5	Extracted RNA from *W. somnifera*	29
3.6	cDNA synthesis using random hexamers	30
3.7	Amplification of L asparaginase gene from cDNA	31
3.8	Purified band of L asparaginase gene	31
3.9	Net charge vs pH graph	33
3.10	Hydropathy plot	33
3.11	Secondary structure of L asparaginase	35
3.12	Secondary structure map of L asparaginase	36
3.13	Intrinsic disordered profile	36

LIST OF ABBREVIATIONS

Symbols	Abbreviation
A	Adanine
ASCT1	Alanine, Serine, Cysteine Transporter
ASNsynt	Asparagine Synthetase
Asp	Asparagine
ATF4	Activating Transcription Factor 4
bp	base pair
BLAST	Basic Local Alignment Search Tool
C	Cytosine
˚C	Centigrade
CHOP	Cyclophosphamide, Hydroxyldaunorubicin, Oncovin
CTAB	Cetyltrimethyl ammonium bromide
C/EBP	CCAAT/enhancer-binding protein
ddH_2O	Double distilled water
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylene diamine tetra acetate
Et.Br	Ethidium bromide
eIF2	Eukaryotic Initiation Factor 2
˚F	Fahrenheit
G	Guanine
GADD	Growth Arrest and DNA Damage induced gene
GCN2	General Control Nonderepressible 2
KDa	Kilo Dalton
Km	Michaelis constant
L	Ladder
Lys	Lyasine
mg	Miligram
ml	Mililitre

mM	milli Molar
$Mgcl_2$	Magnesium Chloride
mRNA	Messenger RNA
mTOR	Mammilian Target of Rapamycin
µM	Micromole
µl	Microlitre
ng	Nano gram
Nuc	Nucleophile
PARP	poly(ADP-ribose) polymerase
PCR	Polymerase Chain Reaction
PEG	Polyethyleneglycol
Pm	Pico mole
rpm	Revolution per minute
Ser	Serine
SDS	Sodium Dodecyl Sulphate
SNAT2	Sodium-Coupled Neutral Amino acid Transporter
T	Thyamine
Taq	*Thermus aquaticus*
T.B.E	Tris borate EDTA
T.E	Tris EDTA
Thr	Threonine
UTR	Untranslated Region
UV	Ultra violet
%	Percentage

ABSTRACT

Probing anticancer genes in medicinal plants is important for their appreciable use in cancer therapy. In the present study three species (*Withania somnifera, Withania coagulans, Solanum lycopersicum*) of family Solanaceae were studied for the anticancer gene(s) encoding L asparaginase, an enzyme used in the treatment of Childhood Acute Lymphoblastic Leukemia (ALL). This enzyme has been reported in a variety of legume species but rather scarcely in family Solanaceae. This study focused on the gene responsible for coding the enzyme, revealed successful amplification of DNA product of ca. 840bp in *Withania somnifera.* The product was amplified, sequenced. Further sequence characterization and translation carried out was: the amino acid percentage, secondary structure, physiochemical properties, intrinsic disordered profile, hydropathy plot, biological and molecular functions. For this purpose, different bioinformatics tools were employed such as: FFPred, PSIPRED, DISOPRED, Peptide Property Calculator and Expasy. The presence of helix and sheet regions depicted that the enzyme being assessed in *Withania* was highly stable. Further, the characterization through GenTHREADER, revealed the pairwise energy of the enzyme aligned with Hydrolase (PDB id 3c17) which was found highest i.e. -552.9 and most favourable. The number of aligned amino acids was highest too (i.e. 256 amino acids), predicting the most promising conformation. Therefore, the data analyses depicted that the enzyme from *W. somnifera* may be employed in the treatment of Acute Lymphoblastic Leukemia. As a future recommendation, it is suggested that the efficacy of enzyme extracted from *W. somnifera* may be tested through clinical trials.

INTRODUCTION

L asparaginase is an enzyme used for the treatment of Childhood Acute Lymphoblastic Leukemia (ALL). Biochemically, it hydrolyses the asparagine into aspartic acid and ammonia. Leukemia cells require huge amount of asparagine to maintain their malignant growth. So to meet this demand they get this non acidic, hydrophilic amino acid both from blood serum as well as the limited amount of amino acid they synthesize (Narta *et al*., 2007b). L asparaginase exploits this high demand the leukemia cells have for asparagine and depletes the circulating pool of asparagine from blood serum. So the leukemia cells are killed by depriving them of the amino acid. The presence of L asparaginase has been documented in a variety of organisms such as animals and bacteria (Howard & Carpenter, 1972a), fungi (Sarquis *et al*., 2004b), actinomycetes (Sudhir *et al*., 2012), algae (Paul, 1982) and plants (Oza *et al*., 2011b) but the antineoplastic potential of the drug has been best elucidated from bacterial source specifically from *E. coli* and *Erwinia caratovora*.

In order to use L asparaginase as an ideal candidate for treatment of ALL it should possess no glutaminase activity, high affinity for its substrate asparagine (low Km value), no side effects, no immunogenic complications and delayed clearance from plasma (prolonged half-life) (Nagarethinam *et al*., 2012b) so as to avoid frequent administration. L asparaginase from bacterial source contain up to 10% L-glutaminase activity with the least activity of 0.01% for *Helicobacter pylori* L asparaginase (Campbell *et al*., 1967a) (Cappelletti *et al*., 2008). The glutaminase activity of L asparaginase is probably due to reason that both asparagine and glutamine have same structural formula possessing amide groups but the difference is that glutamine has one additional methyl group (Ramya *et al*., 2012) and during biosynthesis of asparagine, transamidation of aspartate occurs in which glutamine serves as the amide group donor (Nagarethinam *et al*., 2012b). L asparaginase from Green Chilies also has Glutaminase activity up to 50% and in addition it also possesses urease activity (Bano & Sivaramakrishnan, 1980) which indicate the unreliability of the enzyme in ALL therapy . L asparaginase from *Withania somnifera* have been identified and cloned in *E. coli* and its glutaminase activity is also determined to be 2 % (Oza *et al*., 2011b) but the clinical application of the drug from this source are still awaited. So identification of novel sources for the drug possessing least glutaminase activity is in progress.

Up to my knowledge among the bacterial sources, the lowest Km value of 4.9 x 10 $^{-6}$M has been reported for *Aeromonas spp* L asparaginase (Pattnaik *et al.*, 2000).The enzyme from *E.coli* and *Erwina carotovora* has gained worth in clinical applications against ALL, with Km values of 1.25 x 10^{-5}M and 1.8 x 10^{-5}M respectively (Cedar & Schwartz, 1967, Maita *et al.*, 1979, Shifrin *et al.*, 1974) .Fungi such as *Candida utilis*, *Sacharomyces cereviseae*, *Pichia polymoroha, Penecillium spp, Aspergillus aculeatus, Fusarium tricinctum, Penecillium digitatum* and *Aspergillus terrus* also synthesize L asparaginase with the least Km value of 1x 10^{-5}M reported for *Penecillium digitatum* (Shrivastava *et al.*, 2012). The Km value of the enzyme purified and characterized from *Streptomyces* sp. (an actinomycete) is 2.4 x 10^{-5}M which is comparable to that of *E.coli* and *Erwinia carotovora* (Basha *et al.*, 2009). The optimum pH of the enzyme from this source is 7.5 which is very close to human blood pH but the enzyme shows optimum activity at 50˚C which is far higher than human body temperature. The Km value for the enzyme characterized from *Chlaymydomonas* sp. (an algae) is relatively high i.e. 1.34 x 10^{-4}M and thus showed a little antitumour activity when applied on Gardner, e lymphosarcoma in C_3H mice (Paul, 1982). Plant species such as *Withania somnifera, Capsicum annum, Lupinus arboreus, Lupines angustifolius, Cicer arietinum, Pisum sativum*, *Arabidopsis thaliana* etc. are also potent sources of L asparaginase with the lowest Km value of 7.0 x 10^{-5}M for the enzyme (Recombinant enzyme, WsA) reported from *Withania somnifera* (Oza *et al.*, 2011b).

Leucopoenia, neurological seizures, anaphylaxis, coagulation abnormalities and pancreatitis are the common side effects of L asparaginase. Alves et al in 2007 have reported the incidence of Diabetes Mellitus in patient with lymphoblastic leukemia while treating with L-asparaginase and dexamethasone (Alves *et al.*, 2007). An 18 year young girl victim of acute lymphoblastic leukemia was also reported to have incidence of superior vena cava syndrome and anaphylaxis after induction with PEG L asparaginase (Creel *et al.*, 2008). A child of Thai origin was reported to develop pancreatic panniculitis after administration with L asparaginase (Chiewchengchol *et al.*, 2009). The administration of the enzyme to children suffering from acute lymphoblastic leukemia brought about enhancement in triglyceride and cholesterol levels (Cohen *et al.*, 2010). Lipid abnormalities return to normal in children upon completion of the asparaginase treatment.

The immunogenic complications, resistance and short half life (early clearance from plasma) of the native forms from bacterial source make its clinical applications a nut to crack. Therefore in mid 1970 several groups started chemical modification of L asparaginase by adopting various methods so as to identify the form which was less immunogenic having prolonged half life and retained good antitumor activity. Among the chemical modifications made to L asparaginase, PEGylation (conjugation of L asparaginase to PEG) has been proved to be the most effective method of modification. PEG asparaginase has half-life of 357 hrs compared to native form of E. coli asparaginase which is 20 hrs (Keating *et al.*, 1993). Moreover PEG asparaginase remains in blood serum for more than 28 days when applied at dosage of 2500 IU/mg but in contrast the native form from *E. coli* depletes in 7-8 days (Asselin *et al.*, 1993, Ho *et al.*, 1986, Albertsen *et al.*, 2001). Also lower incidence of complications are associated with PEG L asparaginase (Ettinger *et al.*, 1995).

1.1 Review of Literature

L asparaginase converts L asparagine to aspartic acid and ammonia. Lang (1904) was the first who observed the amido-hydrolytic activity of L-asparaginase and Kidd (1953) explored the anticancer potential of the enzyme by observing the antilymphoma activity of guinea pig serum(Kidd, 1953). Later on the metabolic difference between the normal and malignant cells was determined by Maxwell and Neuman (1956) in the presence and absence of asparagine(Maxwell *et al.*, 1956). Taking clue from all these studies, the anti-lymphoma activity of guinea pig serum to the depletion of asparagine by the enzyme was related by Broome in 1961 (Nagarethinam *et al.*, 2012b). Yellen and Wriston in 1966 carried out partial purification of two isoforms of L asparaginase from guinea pig serum (Yellin & Wriston, 1966b) however only one of them showed anti lymphoma activity (Yellin & Wriston, 1966a). The extraction of enzyme in bulk quantity from Guinea pig serum was difficult so other alternative sources such as microbes were searched out (Narta *et al.*, 2007a). The purification of L asparaginase from *E. coli* and its tumouricidal activity similar to that of Guinea pig serum provided the practical base for its large scale production for pre-clinical and clinical studies (Mashburn & Wriston, 1964), (Campbell & Mashburn, 1969), (Campbell *et al.*, 1967b), (Ho *et al.*, 1970b, Roberts *et al.*, 1966, Whelan & Wriston, 1969). Two asparaginases i.e. EC-1 (cytoplasmic) and EC-2 (periplasmic) were found to be produced by E.coli and anti-lymphoma

activity was exhibited by EC-2 only. The role of the enzyme in the inhibition of human leukemia was for the first time reported by Oettgen et al in 1967(Oettgen et al., 1967). Several clinical trials in patients affirmed the clinical efficacy of L asparaginase and was finally approved as drug for leukemia by FDA in 1978 (Nagarethinam *et al.*, 2012a).

1.2 Molecular Structure

L asparaginases from most of the bacteria have similarity in their biochemical properties, tertiary and quaternary structures (Ramya *et al.*, 2012). Swain et al (1993) and Lubkowski *et al.* (2003) determined the crystal structure of L asparaginase isolated from *E.coli* and atomic resolution structure of the enzyme isolated from *Erwinia chrysanthemi* respectively(Swain et al., 1993, Lubkowski et al., 2003). L asparaginase from different sources has been reported to possess four subunits with each subunit containing the active site. The subunits are arranged in eight monomers with active site lying between two adjacent monomers (A and C; B and D). Four identical subunits (denoted as A, B, C and D) constitute a tetramer. The whole molecule is considered as dimer of dimmers (Jaskolski *et al.*, 2001, Sanches *et al.*, 2003). Each active site is formed by the distribution of amino acids in two adjacent monomers. The active site consists of Thr15, Tyr29, Ser62, Glu63, Thr95, Asp96, Ala120, Lys168 with only Ser254 active site residue located in the adjacent monomer (Jaskolski *et al.*, 2001, Asselin *et al.*, 1999, Eden *et al.*, 1990, Kabsch, 1993, Carlsson *et al.*, 1995, Goldberg *et al.*, 2003, Avramis & Tiwari, 2006, Vieira Pinheiro *et al.*, 2006, Narta *et al.*, 2007a, Janin *et al.*, 2006, Park *et al.*, 1981, Ramya *et al.*, 2011). Thr15 and Thr95 are the key catalytic residues among the aforementioned residues.

1.3 Mechanism of action

The substrate of L –asparaginase, L-asparagine is from amide group amino acids and is the paramount nutrient for tumor cells. Due to enhanced rate of proliferation and metabolic process taking place in cancerous cells, they require elevated level of the amino acid (Luhana *et al.*, 2013). So meet the demand they get asparagine in two ways i.e. from blood serum and what they synthesize themselves which is very limited. L asparaginase as a drug exploit this and hydrolyses L asparagine into aspartic acid and ammonia thereby killing the tumour cells by

depriving them from this amino acid (Narta *et al.*, 2007b). The low level of asparagine only affects the viability of the cancerous cells compare to the normal cells. Healthy cells however have high amount of Asparagine synthetase and escape from this phenomenon (Narta *et al.*, 2007b). The transformation of L asparagine to L aspartate and ammonia by L-asparaginase involves a double displacement or "Ping Pong" mechanism in which nucleophilic group of the enzyme attacks the C_γ of the substrate asparagine forming a tetrahedral intermediate which thereafter breaks down to form an acyl-enzyme intermediate. This is followed by elimination of ammonia. Then a second nucleophile (normally water) attacks this intermediate transforming it into acidic product (i.e Aspartic acid) and free enzyme (Ehrman *et al.*, 1971, Röhm & Van Etten, 1986)

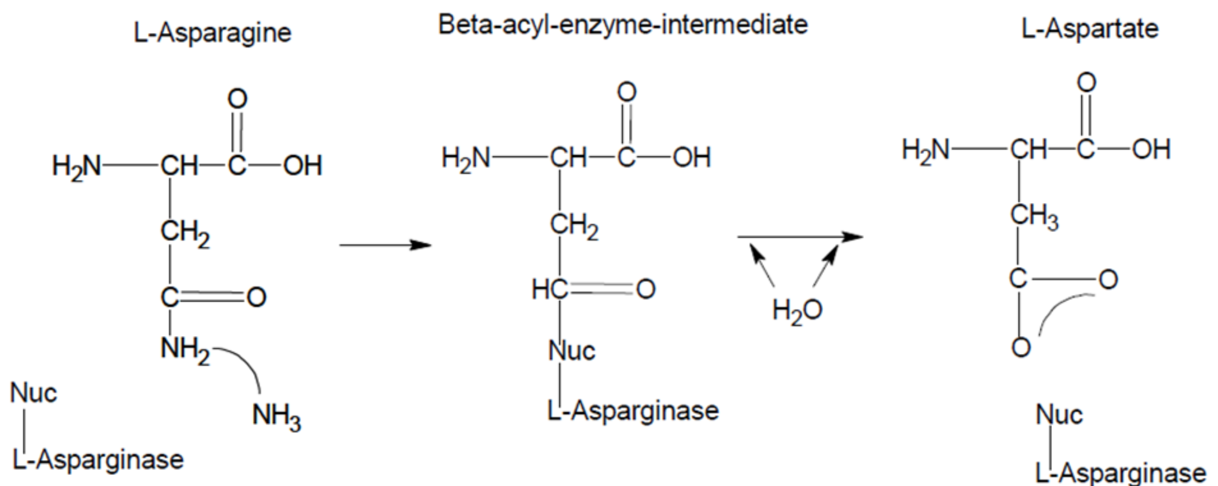

Fig 1.1 Asparagine degradation by L asparaginase

Asparagine is important in the biosynthesis of protein, DNA and RNA and cells need asparagine in order to proceed through G1 cycle of cell division (Michael Rytting, 2012). The inability of leukemia cells to synthesize L asparagine de novo is related to lack or presence of very low levels of asparagine synthetase in them (Kiriyama *et al.*, 1989, Prager & Bachynsky, 1968), however (Stams *et al.*, 2003) have strongly contradicted this and stated that the sensitivity to L asparaginase is not associated with expression levels of asparagine synthetase.

1.4 Metabolic pathways triggered by Asparaginase

Since asparaginase breakdown asparagine and glutamine, nutritional stress is created in the treated cells which is reflected by metabolic responses (Covini *et al.*, 2012). Both the amino acids are nitrogen transporter and their relative importance vary from organism to organism with glutamine appearing much more important than asparagine (Covini *et al.*, 2012). The most abundant amino acid in human plasma is glutamine and its concentration lies between 0.6 – 0.9 mM while in contrast the concentration of asparagine is 10 to 15 times lower (Cooney *et al.*, 1970). Gln is also the most abundant amino acid in intracellular compartment however higher variability exists for its concentration among different tissues (Souba, 1993). Glutamine is also inevitable in culture media for in vitro growth of mammalian cells while these media are nominally asparagine free (Eagle *et al.*, 1956). However no solid evidence has been provided for this requirement because glutamine is non-essential amino acid and most of the cultured cells synthesize it de novo. So due to these reasons depletion of glutamine by asparaginases have been studied extensively than asparagine depletion (Covini *et al.*, 2012).

However the depletion of asparagine induces ASNS (Asparagine Synthetase) gene (Gong *et al.*, 1991) . But the induction is not confined to this condition only, as the induction of corresponding amino acid synthetase can be observed upon the depletion of any amino acid(Gong *et al.*, 1991). Under such circumstances the GCN2 kinase is activated in cells which bring about the phosphorylation of α-subunit of eIF2α. The phosphorylation acts as a fundamental metabolic switch that slows down the protein synthesis. So under such conditions cell conserves energy for its survival (Covini *et al.*, 2012).

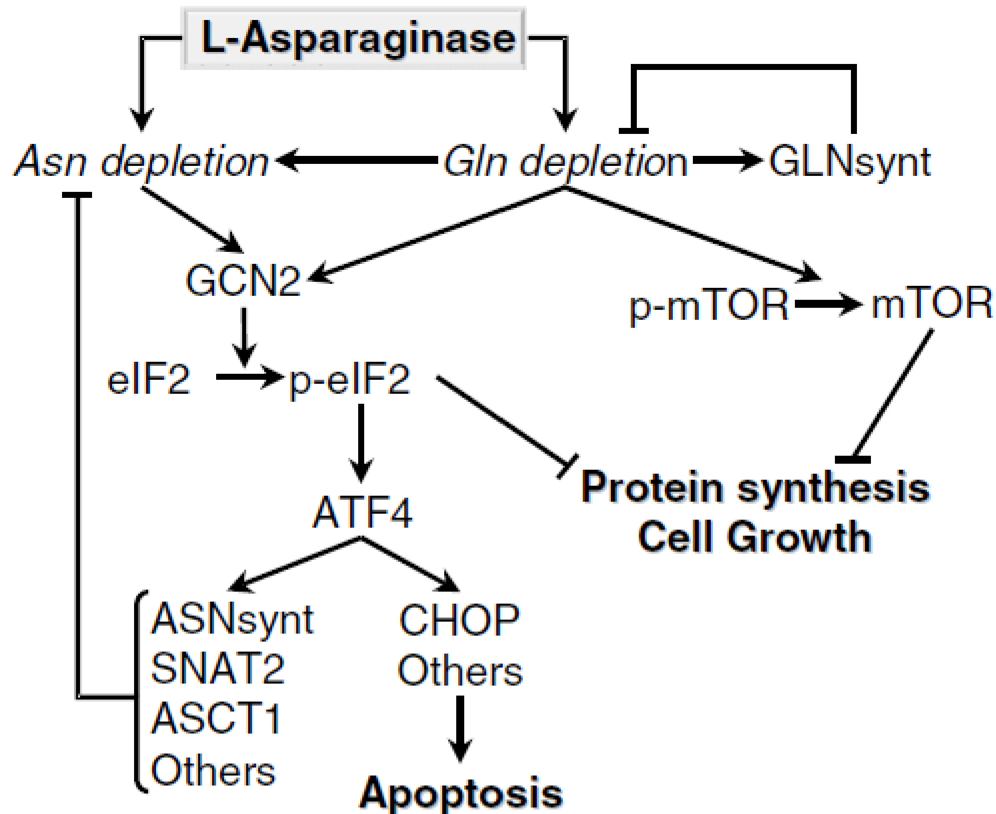

Fig 1.2: Schematic diagram for metabolic pathways triggered by L asparaginase

In the meantime mRNA enriched with IRES (Internal Ribosomal Entry Sites) are translated into proteins involved in cell defense or proteins involved in cell death (Covini *et al.*, 2012). For example one such protein is Transcription Factor ATF4, the translation of which activates many genes responsible for cell survival or depending upon the environmental condition trigger apoptosis such as GADD153/CHOP.

Several metabolic pathways drawn out by Asparaginase treatment are directly related to nutritional stress caused by asparagine and /or glutamine depletion. For example, when MOLT-4 leukemia cells are treated with L asparaginase from *E.coli*, then along with ASNS gene induction, two Na-dependent transporters (i.e. ASCT1 and SNAT2 for asparagine and glutamine respectively) are also induced (Aslanian & Kilberg, 2001). Moreover since L asparaginase also exhibits Glutaminase activity, Glutamine Synthetase gene is also induced upon asparaginase treatment. Fine *et al* in 2005 reported more than 800 genes which modified their expression after treatment with asparaginase (Fine *et al.*, 2005). Among these, the gene

encoding ASNS, tRNA synthetases, amino acid transporters, ATF and CCAAT/enhancer-binding protein (C/EBP) families of transcription factors were found induced while the genes involved in proliferation were suppressed. Interestingly, ATF4 and members of the C/EBP family synergistically interact to promote induction of amino acid-responsive genes by binding to composite C/EBP-ATF response elements (CARE) (Kilberg *et al.*, 2009). The drawbacks of the enzyme in ALL therapy arise because of the fact that the drug has several side effects and the patients show immunogenicity as a consequence, resistance to the drug occur.

1.5 Side effects of L-asparaginase

Most common side effects associated with L asparaginase therapy include imbalances in the formation of clotting factors such as plasminogen (Priest *et al.*, 1980) , protein C and protein S (Homans *et al.*, 1987, Barbui *et al.*, 1983, Vigano'D'Angelo *et al.*, 1990, Trivedi & Pitchumoni, 2005) and antithrombin III (Aoki *et al.*, 2005, Brodtman *et al.*, 2005, Sahoo & Hart, 2003b). Acute pancreatitis is also one of the side effects which was well documented complication involved in leukaemia therapy; this resembles drug-induced pancreatitis in most cases and the symptoms associated with them include vomiting, abdominal or back pain, anorexia (Imamura *et al.*, 2005). Patients receiving intensive L-Asparaginase therapy were found to be affected with myocardial infarction and also have a chance of developing secondary leukaemia which can be induced as a result of topoisomerase targeted drugs. Problems associated with immunodeficiency and acute hepatic dysfunctions are the major side effects of L- Asparaginase in leukemia therapy (Haddy *et al.*, 2006, Hernandez-Espinosa *et al.*, 2006). During ALL therapy, onset of venous thrombosis in children has been reported (Sahoo & Hart, 2003a). In adolescent patients with leukaemia, due to L asparaginase therapy, cerebral thrombotic complications were observed (Mitchell *et al.*, 1994).

Growth hormone deficiency particularly in children (Omoti & Omoti, 2006) and increased risk of thrombosis was observed in patients treated for ALL (Saviola *et al.*, 2004). Thromboembolic problems in paediatric patients with ALL were due to the poor regulation of thrombin and prothrombin levels in the blood after the therapy with the enzyme L-asparaginase (Ortega *et al.*, 1977). L asparaginases were found to be associated with corneal toxicity in patients given with combinational chemotherapy. Foreign body sensation, blur red vision, ocular pain, bilateral conjunctival hyperaemia were found to be the common symptoms

observed (Sutow *et al.*, 1971). In patients with ALL, due to L- asparaginase therapy, myocardial ischemia has been reported. Even symptoms related to diabetes were seen due to damage of islet cells of pancreas and subsequently, insulin levels are decreased (Oettgen *et al.*, 1970, Zollner & Heimstadt, 1971). Simultaneous occurrence of parotitis and abnormalities in lipid metabolism were also observed during L asparaginase therapy (Favrot *et al.*, 1984, Steinherz, 1994, Meyer *et al.*, 2003, Parsons *et al.*, 1997).

Early reports showed that there is a decrease in the level of serum cholesterol and triglycerides in most patients. Later, hypolipidemia followed by hyperlipidemia were also reported (Land *et al.*, 1972). In patients with T-cell lymphoblastic lymphoma, L-Asparaginase associated hyperlipidemia with hyperviscosity has been reported by Meyer *et al* in 2003 (Meyer *et al.*, 2003). Several reports show that hypertriglyceridemia was observed mostly in children and in very few cases in adults undergoing L asparaginase therapy (Oettgen *et al.*, 1970, Ohnuma *et al.*, 1969). Impairments of CNS functions along with agitation, hallucinations, disorientation, convulsions and coma were observed (Korholz *et al.*, 1987). Korholz et al. reported that there is an increase in the symptoms of nervous disorders after the administration of L-asparaginases. Asparaginases were found to elicit hypersensitive reactions, and these reactions are due to the production of high titres of IgG3 antibodies associated to a higher risk of anaphylaxis. Many reports clearly indicate that E. coli L asparaginase causes more hypersensitive reactions when compared to *Erwinia spp*.

1.6 Immunogenic complications/Resistance to the Drug

Different researchers have presented different opinions about the resistance to L asparaginase (Ho *et al.*, 1970a). Worton *et al.*, (1991) have reported that resistance to the drug is primarily due to depression of Asparagine Synthetase gene. Gene silencing approach may be helpful in the inactivation of asparagine synthetase gene. (Capizzi, 1993) have related the resistance to the production of specific antibodies against the drug. Gallagher et al 1989 opinioned that the cells that are sensitive to L asparaginase produce cytokines. These cytokines control the expansion of resistant cells. As soon as the sensitive cells are killed by L asparaginase,the resistant cells escape from regularly control. Airoldi et al 2011 have reported that cytokines (IL 12 family members) have direct anti-tumor activity against B-ALL. They also reported that the cytokines, G-CSF and GM-CSF activate quiescent leukemia cells,

stimulate their proliferation and make them sensitive to chemotherapeutic drugs. Kullas et al. (2012) have different opinion. They reported that L asparaginase suppresses the production of cytokines. Holleman *et al* (2003) associated the resistance to L asparaginase and prednisolone to inactivation of capase 3 or PARP [poly (ADP-ribose) polymerase]. Ehsanipour *et al* (2013) have documented that Adipocytes which produce sufficient quantities of Glutamine cause the Leukemia cells to resist against L asparaginase. All these limitations probably have arisen from glutaminase side activity. Various attempts have been employed to eradicate its side effects.

1.7 Chemical Modification of the Enzyme

Three preparations i.e. two native and one modified form (from either of the native forms), of L asparaginase used in clinics are available. The native form of the enzyme has been isolated from *Erwinia caratovora* and *E.coli*. The molecular weight of the enzyme isolated and purified from various strains of E.coli range from 132-141KDa (Irion & Arens, 1970, Jackson & Handschumacher, 1970; Maita & Matsuda, 1980) while the molecular weight of *Erwinia* L asparaginase turns out to be 138 KDa. The purified enzyme has specific activity lies between 300-400 µmol of substrate/mg protein. The pH range for isoelectric point of the enzyme ranges between 4.6 and 5.5 for E. coli while 8.7 for L asparaginase from *Erwinia* (Howard & Carpenter, 1972b). The approximate Km value for l-asparaginase $1\times10{-}5$mol/l (Jackson & Handschumacher, 1970, Maita & Matsuda, 1980). These asparaginases contain up to 10% glutaminase activity which is undesirable. Among the chemical modification made to L asparaginase, PEGylation (conjugation of L asparaginase to PEG) has been the most reliable method applied to L asparaginase modification. The enzyme was successfully conjugated to PEG for the first time in 1979 by Abuchowski *et al*. The antileukemic properties of this modied enzyme were tested on L5178Y tumor bearing BDF mouse model and the coupling succeeded in abolishing the drugs immunogenicity. The PEGylation resulted in markedly different biochemical properties from that of native forms. Its apparent molecular weight was higher and its affinity to react with specific antibodies was very low which increased when the drug is subjected to freeze thawing cycles (Koerholz *et al*., 1989). This modified form has the antineoplastic activities both in animal models(Abuchowski *et al*., 1979, Yoshimoto *et al*., 1986) and human (Jürgens *et al*., 1988) and has several advantages over the native forms. Patients treated with the modified form of the enzyme have diminished incidences of

hyperglycemia and pancreatitis and absence of anaphylaxis (Ettinger *et al*., 1995). It has prolonged half life and requires less frequent administration. It is administrated biweekly as opposed to 2-3 medications a week of native E.coli preparation (Narta *et al*., 2007b). The actual cost to the patients for peg-asparagase is greater than that of native froms but the less frequent visit of Physician and the reduced incidence of immunogenic complications make the overall treatment cost considerably less than that of native preparations (Narta *et al*., 2007b). PEG L asparaginase is commercially available as pegasparagase by generic name; and ONCASPAR is the trademark of the manufacturer, ENZON, South Plainfield, NJ. Comparison of native and modified L asparaginases is given in Table 1.1.

Table 1.1 Comparison of native and modified L asparaginase

	E.coli		***Erwinia* (Native)**
	Native	**PEGylated**	
Activity(IU)/mg protein	280-400	280-400	650-700
*K*m (µM)-l-asparaginase	12	12	12
Km (µM)-l-glutaminase	3000	3000	1400
l-Glu/l-Asp(maximal activity)	0.03	0.03	0.10
Molecular weight	141000	-	138000
PI	5.0	5	8.7

Several other chemical modifications of the enzyme with their outcomes are summarized in Table 1.2

Table 1.2 Various Chemical modifications of L-asparaginase

Chemical modification	**Outcome**
PEG L asparaginase	The drug,s immunogenicity was abolished (Yoshimoto *et al.*, 1986)
L asparaginase with Dextran	Less effective immunogenicity reduction than with PEG(Davis *et al.*, 1991)
L asparaginase with poly-dl-alanyl peptides	Clinical studies have not been reported (Narta *et al.*, 2007).
Acylation	The enzyme becomes hydrophobic (Martins *et al.*, 1990)
Palmitoyl l-asparaginase	10 fold prolongation in half life without acute toxicity (Jorge *et al.*, 1994)
L asparaginase with polyoxyethylene allyl methyl diether	Immunoreactivity towards anti-asparaginase serum was lost completely (Yoh Kodera *et al* 1992)
Silk fibroin-L-ASNase bioconjugate	Lower immunogenicity and antigenicity compared with the native enzyme (Zhang *et al.*, 2005) and has increased thermal stability (Wang & Cao, 2011).

Polyphosphazenes biopolymers bear two active chlorine side groups on each repeat unit along the biocompatible and biodegradable inorganic backbone(Qiu, 2013). So the conjugation of L aspraginase to this polymer may turn out to be helpful to further improve the pharmacokinetic properties of the drug so that it could be successfully administered to adult patients also. Moreover non-toxic, biodegradable, biocompatible polymers from medicinal plants may also serve to be fruitful in this regard.

1.8 Sources of L-asparaginase

On the basis of amino acid sequence and biochemical properties,the enzyme possessing asparaginase activity can be categorized into several families (Borek & Jaskolski, 2001), however Bacterial and Plant-type asparaginases are the largest and best characterized families (Oza *et al.*, 2011a). Here L asparaginase from different sources such as Bacteria, Fungi,

Actinomycetes, Algae and Plants, with their biochemical properties that are considered to be important in cancer therapy will be discussed.

1.8.1 Bacterial L-asparaginase

In bacteria two types of L-asparaginase i.e Type 1 and Type II have been reported (Campbell *et al.*, 1967b). Type1 are expressed in cytoplasm and hydrolyse both asparagine and glutamine while Type II are expressed in periplasm and require anaerobic environment for their expression. Type II L-asparaginases have high affinity for L-asn which is the main nutrient for tumor cells. The molecular mass of different native Asparaginases from Bacteria varies between 140-160 KDa.The presence of L asparaginase has been reported from many bacteria such as *E.coli*, *E. carotovora*, *Bacillus sp*, *Pseudomonas spp* etc. L-asparaginase from halophilic bacteria is expected to be non-allergic and hence halophilic bacteria from saline soil can contribute to therapeutic value of this enzyme (Kamble *et al.*, 2012). Some of the bacterial L-asparaginase with their biochemical properties are summarized in Table 1.3.

Table 1.3 Biochemical properties of some bacterial Asparaginase

Bacteria	Biochemical properties				
	pH optima	Temp Optima (°C)	Km (M) L-asparagine	M.W (kDa)	Reference
Pseudomonas aeruginosa	9	37	$0.147_{x}\ 10^{-3}$	160	(El-Bessoumy *et al.*, 2004)
Proteus Vulgaris	7-8	57	$2.6_{x}\ 10^{-5}$	-	(Tosa *et al.*, 1972)
Vibrio succinogenes	7.3	-	4.78×10^{-5}	146	(Distasio *et al.*, 1976)
Bacillus coagulans	8.5-9.5	55	4.7×10^{-3}	85	(Law & Wriston, 1971)
Mycobacteriun phlei	8.8-9.2	-	0.7×10^{-3}	126	(Rozalska, 1989)
Erwinia carotovora	8	50	1.8×10^{-5}	125-145	(Maladkar *et al.*, 1993, Cammack *et al.*, 1972)
Erwinia aroideae	7.5	-	3×10^{-3}	155	(Peterson & Ciegler, 1969, Tiwari & Dua, 1996)
E.coli	7-8	37	1.25×10^{-5}	141	(Cedar & Schwartz, 1967, Shifrin *et al.*, 1974)
Azotobacter vinelandii	8.6	48	1.1×10^{-4}	84	(Gaffar & Shethna, 1977)
Pseudomonas fluorescens	8-9	-	4.1×10^{-4}	70	(Nilolaev *et al.*, 1975)
Pseudomonas stutzeri	9	37	1.45×10^{-4}	34	(Manna *et al.*, 1995)
Acinetobacter calcoaceticus	8.6	-	2×10^{-3}	105	(Joner *et al.*, 1973)
Bacillus subtilis B11-06	7.5	40	4.3×10^{-4}	38 each subunit	(Jia *et al.*, 2013)
Corynebacterium glutamicum	7	40	2.5×10^{-3}	81	(Mesas *et al.,* 1990)
Helicobacter pylori	7-10	45	2.9×10^{-4}	140	(Cappelletti *et al.*, 2008)
Aeromonas spp	9	50	4.9×10^{-6}		(Pattnaik *et al.*, 2000)

Cappelletti *et al.*, 2008 have reported a new L asparaginase from Helicobacter pylori. The enzyme from this source has the lowest glutaminase activity i.e 0.01% and require optimum pH of 7-10 for its asparaginase activity. But as evident from the table, its Km value is relatively higher than E.coli L asparaginase and its higher cytotoxic effects have also been reported by the same group. Recombinant ASNase derived from W. succinogenes also has a

very low activity towards Gln and this trait may result in prominent reduction in side effects and it also has a low cross reactivity. So the efficacy of the enzyme in treatment of ALL from this source need to be evaluated.The enzyme from pseudomonas sp is GLN-ASNase. Although it is stable and has prolonged half life in tumor-bearing hosts but it has low Km value (i.e 2.2 x 10^{-5} M) for glutamine which is undesirable and hence this character would probably limit its apllication in cancer therapy.

1.8.2 Fungi L-asparaginase

L-asparaginase is also efficiently produced by fungi and the enzyme from fungal source have less side effects in comparison to bacteria (Sarquis *et al*., 2004). The production of L-asparaginase by Filamentous fungi viz. *Aspergillus tamarii* and *Aspergillus terreus* have been reported to produce L asparaginase with the highest production in 2% proline medium from A. terreus (Sarquis *et al*., 2004a). Ali *et al* (1994) have reported that *A.terrus* L-Asparaginase was non toxic,myelosuppressive and immunosuppressive. L- asparaginases that are currently in use have been reported from a variety of Yeasts specially *Saccharomyces cervisiae* and is encoded by the ASP3 gene (Bon *et al*., 1997). The production of L- asparaginase has also been reported from *Pichia polymorpha*, was isolated form Egyptian Soils by Enrichment method (Foda *et al*., 1980). The enzyme has also been isolated from the cell culture broth of *Candida utilis* (Kil *et al*., 1995). The enzyme from candida utilis has low Km value but it has less affinity towards L-asparagine and clears from plasma at faster rate indicating its inferior antitumor potential compared to *E.coli* (Sakamoto *et al*., 1977). The less antitumor potential of the enzyme from this source is probably due to presence of mannan moeity in it. Some of the biochemical properties of fungi L- asparaginase are summerized in Table 1.4.

Table 1.4 Biochemical properties of Fungi L- asparaginase

Fungi	Biochemical Properties				
	Opt pH	Opt Temp (°C)	Km(M)	M.W (KDa)	**Reference**
Saccharomyces cervisiae	6.8	-	3.5 x 10^{-4}	800	(Dunlop *et al.*, 1978)
Pichia polymorpha	6.7	-	1.37 x 10^{-2}	-	(Foda *et al.*, 1980)
Rhizomucor miehei	7	45	-	133.7	(Huang *et al.*, 2013)
Penecillium digitatum	7	30	1 x 10^{-5}	-	(Shrivastava *et al.*, 2012)
Candida utilis	-	-	7.7 x 10^{-5}	480	(Sakamoto *et al.*, 1977)
Penecillium spp	7	37	4 x 10^{-3}	66	(Patro & Gupta)., 2012
Fusarium tricinctum	7.5-8.7	-	5.2 x 10^{-4}	161-170	(Scheetz *et al.*, 1971)
Aspergillus terrus	5-7	40-45	5.8 x 10^{-4}	-	(Ali *et al.*, 1994)
Cylindrocarpon obtusisporum	7.4	37	1 x 10^{-3}	216	(Raha *et al.*, 1990)
Cladosporium sp	6.3	30	1 x 10^{-1}	121	(Kumar & Manonmani, 2013)
Aspergillus niger	6	37	1.6 x 10^{-5}	-	(Luhana *et al.*, 2013)

Limited studies are available on the evaluation of the enzyme from fungal sources in the treatment of ALL.

1.8.3 Actinomycetes L-asparaginase

There are more than 22,000 known microbial secondary metabolites, 70% of which are produced by actinomycetes and among the actinomycetes, streptomycetes group are considered economically important because out of the approximately more than 10,000 known antibiotics, 50–55% are produced by this genus (Subramani & Aalbersberg, 2012). Much research is being done on these organisms for they have gained attention as rich sources of antibiotics, anti-

tumour drugs and other bioactive molecules(Sudhir *et al.*, 2012). Actinomycetes are the least studied organisms among all the L-asparaginase producing sources (Sudhir et al., 2012). Among the actinomycetes, several Streptomyces species such as *S. karnatakensis, S. venezualae, S. longisporusflavus, S. ginsengisoli* (Deshpande *et al.*, 2014) and a marine Streptomyces sp. PDK2 have been reported to produce L-asparaginase (Mostafa & Salama, 1979, Mostafa, 1979, Abdel *et al.*, 1998) Dhevagi and Poorani, 2006). There are also reports of L-asparaginase production from some Streptomyces sp. isolated from the gut of the fish *Therapon jarbua* and *Villorita cyprinoides*, *Streptomyces gulbargensis* and *Streptomyces noursei* MTCC 10469 (Dharmaraj, 2011, Amena *et al.*, 2010). Gupta *et al* 2007 have reported marine actinomycetes to be a potential source of L-asparaginase far they have extreme adaptaion within marine environment. Very little attention is paid on L asparaginase activity of marine actinomycetes purely at screening level because of difficulties in their identification and classification. It would be worthwhile to focus on the properties such as optimum pH, optimum temperature for activity, percent glutaminase activity, toxicity and Km value of the enzyme for its substrate L asparagine from actinomycete sources.

1.8.4 Algae L-asparaginase

Micro algae L asparaginase was for the first time purified to near homogeneity by John H. Paul from marine *Chlamydomonas spp*. (Paul, 1982). The molecular weight of the purified enzyme is 275 KDa with Km value for asparagine 1.34×10^{-4} M. It shows some degree of thermal stability and possesses optimum activity over a wide range of pH (6.8-9.52). The enzyme shows less antitumor activity in an antilymphoma assay *in vivo*.

1.8.5 Plant L-asparaginase

L asparaginase has been reported from a number of Plant species such as Barley roots (Grover & Chibnall, 1927), seedling of *Lupinus leutus* and *Dilichos lab* (Lees & Blakeney, 1970), Green chillies (Bano & Sivaramakrishnan, 1980), *Tamarindus indica* (Bano & Sivaramakrishnan, 1980), *Lupinus angustifolius* (Dickson *et al.*, 1992), soybean leaves (Cho *et al.*, 2007), leaves, flowers buds and root tips of *Lupines arabreus* (Lough *et al.*, 1992b), *Arabidopsis thaliana* (Bruneau *et al.*, 2006), cotyledons of chickpea seedlings (El Shora & Ali), immature seeds of pea (Sodek *et al.*, 1980), *Lotus japonicas* (Credali *et al.*, 2011), *Withania*

somnifera, *Datura innoxia*, *Lycpersicum lycopersicum*, *Vigna unguiculata*, *Asparagus officinalis*, *Oryza sativa* (Oza *et al.*, 2009a). L asparaginase activity has also been reported in soil of roots of *Pinus pinaster* and *Pinus radiate* (Bell & Adams, 2004). Some trace amount of the enzyme is also present in *Solanum melongena*, *Arachis hypogea*, *Glycin wightii*, *Saraca asoca*, *Delonix regia*, *Casia fistula* (Oza *et al.*, 2009a).

Though L-asparaginase has been reported in many higher plants, little work has been carried out on the purification and characterization of L-asparaginase from higher plants (Oza *et al.*, 2009b). In plants, L-asparagine is the major nitrogen storage and transport compound, and it may also accumulate under stress conditions (Sieciechowicz *et al.*, 1988). Asparaginases liberate from asparagine the ammonia that is necessary for protein synthesis (Oza *et al.*, 2009). There are two groups of such proteins, called potassium-dependent and potassium-independent asparaginases. Both enzymes have significant levels of sequence similarity (Oza *et al.*, 2009b). The plant asparaginase amino acid sequences did not have any significant homology with microbial asparaginase but was 23% identical and 66% similar to a human glycosyl asparaginase (Lough *et al.*, 1992a, Lough *et al.*, 1992b). The biochemical properties of L asparaginase from some of the plant sources are summarized in Table 1.5.

Table 1.5: Biochemical properties of Plant L-asparaginase

Plants	Biochemical Properties				
	pH	Temp(˚C)	Km(M)	M.W (KDa)	**Reference**
Withania somnifera	8.5	37	$6.1x\ 10^{-5}$	72	(Oza *et al.*, 2009a)
Capsicum annum	8.5	37	3.3×10^{-3}	120	(Bano & Sivaramakrishnan, 1980)
Pisum sativum	-	-	2.4×10^{-3}	69	(Chagas & Sodek, 2001)
Lupinus arabreus	-	-	6.6×10^{-3}	75	(Chang & Farnden, 1981)
Lupinus angustifolius	-	-	7×10^{-3}	75	(Chang & Farnden, 1981)

Only limited studies are available on anticancer properties of the enzyme from plant source. Oza *et al* (2010) reported the antitumor activity of the purified enzyme from *Withania somnifera* on cell cultures (Oza *et al.*, 2010). In the same article they also reported the superiority of the enzyme over bacterial ones because of having less toxic effects on cell cultures. The Km value (for asparagine) of the enzyme cloned and expressed in E. coli from this source came out to be 7.02×10^{-5}M. The recombinant enzyme showed 2% glutaminase activity which is comparable to that of E. coli L-asparaginase. The in vivo use of the enzyme from this source against ALL is still awaited.

1.9 Aims and Objectives

The objectives of this study are as follows:

1- To screen selected members of family Solanaceae for anticancer gene encoding L. asparaginase.
2- To validate the structural and functional role of L asparaginase in cancer therapy using bioinformatics tools.

MATERIALS AND METHODS

2.1 Plant Material

The selected plants species (*Withania somnifera, Withania coagulans, Solanum lycopersicum*) of family Solanaceae were collected from QAU Botanical Garden in May 2013, through prior permission of the Director Botanical Garden. Fresh leaves and Fruits (two weeks old fruits) were selected for DNA and RNA extraction.

2.2 Genomic DNA Extraction

Genomic DNA from the selected plants was extracted using the method described in Doyle and Doyle (1990) with some modifications. About 0.3-0.5 g of leaf and fruit samples were ground in mortar and pestle using Liquid Nitrogen. Thawing of the samples was avoided and the crushed samples were immediately transferred to 1.5ml microfuge tubes. Then about 0.5 ml of freshly prepared pre-warmed CTAB buffer was poured and the tubes were shaken vigorously. The samples were incubated at 65˚C for 40 minutes. During this the tubes were inverted after every 10 minutes. The tubes were then allowed to cool for 5 minutes at Room Temperature and same volume of chloroform:isoamyl alcohol(24:1) was added to separate proteins and DNA in two distinct phases with the lower phase containing proteins and polysaccharides while the upper phase containing the DNA. The tubes were then centrifuged for 7 minutes at 12000rpm to separate the phases. The top aqueous phases were then carefully transferred to new sterile 1.5ml microfuge tubes and equal volumes of chilled isopropanol were added to each tube. The tubes were inverted gently until whitish thread-like DNA appeared. The samples were again subjected to centrifugation for 7 minutes at 12000rpm so as to precipitate the DNA into pellet form at the bottom of the tubes. Isopropanol was then poured off carefully without disturbing the pellet. The pellet was then washed with 1 ml of 70% ethanol and centrifuge for 5 minutes at 12000rpm. The ethanol was then poured off and the pellet was let air dried overnight. The pellet was then resuspended in 50µl PCR water. The dissolved DNA was stored at -20˚C.

2.3 Isolation of total RNA

All the steps in RNA isolation were carried out at 4˚C. About 0.5 grams (g) leaves and immature fruit samples from the selected plants were taken and ground in liquid nitrogen using mortar and pestle. Thawing of the samples was strictly avoided and the samples were quickly transferred to Pre-chilled 1.5ml microfuge tubes. Then 0.5 ml of trizol was added, vortexed and mixed. Samples and trizol were kept in ice all the times. The tubes were then centrifuged for 10 minutes at 12000rpm.The clear top aqueous material was collected in fresh tubes and equal volume of chloroform was added. After careful mixing, the samples were again centrifuged at 4˚C for 15 minutes at 15000rpm.Then about 0.6ml of top aqueous phase was collected in sterile 1.5ml microfuge tubes and precipitated with 0.3ml of chilled isopropanol. The samples were then let at RT for 10 minutes and further centrifuged for 10 minutes at 15000rpm.The supernatant was poured off without disturbing the pellet. The pellet was then washed with 75% ethanol and centrifuged for 10 minutes at 14000rpm. The pellet was then resuspended in 50ul DEPC treated water. The samples were then stored at -20˚C.

2.3.1 cDNA synthesis

Fermentas cDNA synthesis kit was used to synthesize cDNA from RNA samples. All the steps were carried out on cold temperature preferably on ice. Construction of cDNA Libraries were carried out in three steps in PCR. Total reaction mixture of 20 ul was prepared for cDNA synthesis. All the components of the kit were mixed, briefly centrifuged after thawing. The components of the kit were mixed in indicated order mentioned in kit. Firstly 6 ul Nuclease free water was taken in autoclaved 1.5ml microfuge tube and mixed with 1ul Ribolock RNase^{TM} Inhibitor, 5 ul of extracted total RNA, 2 ul of 10mM dNTPs, 4 ul of 5X Reaction Buffer,1 ul of Random Hexamer primer and finally 1 ul of Reverse Transcriptase. After gently mixing and centrifugation for 30 sec, the mixture was placed in PCR. The temperature profile for cDNA libraries construction has been given in the following Table 2.1.

Table 2.1: PCR Temperature profile for cDNA synthesis

S. No	Steps	Temperature(˚C)	Time(minutes)
1	Incubation	25	5
2	Synthesis	42	60
3	Termination	70	5

The dilutions were made by adding 48 ul water and 2 ul of newly synthesized cDNA. The product yielded after RT reaction was used in subsequent PCR immediately. For long term storage, the product was stored at -70˚C.

2.4 Amplification of L asparaginase Gene

2.4.1 Primer Designing

In order to amplify L asparaginase in selected members of the family, primers were designed using both Genomic DNA as well as full length cDNA. The forward and reverse primers were designed for the Target gene in such a way that the forward primer anneals within the 5' UTR and the reverse primer anneals at the 3' UTR region on the sequences obtained from National Centre for Biotechnology Information (NCBI).The primers pair used for amplification of Genomic DNA and cDNA are mentioned in Table 2.2.

Table 2.2 L asparaginase primers designed for Genomic DNA and cDNA

Gene	Type	Primer Sequence
L asparaginase	Genomic DNA	Forward 5/- CTCGTCGTACGTTCTCTTTC-3/ Reverse 5/-GGAATGTTCAGGGCTTGTGC-3/
L asparaginase	cDNA	Forward 5/- ATGGGCGGCTGGAGCATTGC-3/ Reverse 5/- CTTTCAGGCTCAGGCCTTTA-3/

2.4.2 Polymerase Chain Reaction

The PCR conditions for genomic DNA primers were not optimized while for cDNA the conditions were optimized for best amplification results. L asparaginase gene was amplified from cDNA of *Withania somnifera* by using a single set of primers that recognized conserved regions of the gene. PCR amplification was performed with 11.5 ul of PCR water. The reaction was set up in 2.5 ul 10X Taq Buffer, 2 ul of 25mM $MgCl_2$, 0.8ul of dNTPs, 2 ul of 20pm each primer,2 ul of cDNA prep and 0.5 ul of Taq DNA polymerase in a 20 ul of total reaction volume. The PCR procedure comprised 35 cycles of 1 min at 95°C, 1.0 min at 54°C and 1.0 min at 72°C. A final extension time at 72C for 5 min was performed after 35 cycles. The resultant PCR amplicon was sequenced in multicolumn sequencer (ABI, USA). The sequence was validated by BLASTn.

2.4.3 Agarose Gel Electrophoresis of PCR Products

The PCR products were validated by running the samples on agarose gel. For this purpose 2% agarose gel was prepared in TBE buffer. The gel was stained with 2 ul ethidium bromide. Bromophenol blue was added as a loading dye. The visualized bands were compared with Generuler™ 1kB DNA marker (Fermentas) to determine the size of target bands. Electrophoresis was done at 100 V/140 mA for 45 min and visualized by Gel Documentation apparatus equipped with Ultra Violet trans-illuminator.

2.4.4 Elution of DNA Bands from Agarose Gel

Gel Elution Kit (Fermentas, Canada) was used for elution and purification of amplified DNA fragments. The fragment of interest was excised from gel using a sterile blade. The size of the gel slice was minimized without disturbing the fragment. The excised gel band was transferred to 1.5ml microfuge tube and weighed. Add 1ml of binding buffer and incubated for 15 min at 55°C. Brief mixing was repeated by inverting the tubes after every 5 min so that the gel slice was completely dissolved. The mixture was supplemented with 7 ul of silica powder suspension. The suspension was mixed well and allowed to incubate at 50°C for 5 min. The samples were centrifuged at 12000rpm for 30 sec and debris was removed without losing the pellet. Pellet was resuspended in 500 ul of ice cold silica washing buffer. Vigorous shaking and harsh pipetting was avoided. Centrifuge for 1 min, again pellet was form and debris was

discarded. Washing with 500 ul of silica washing buffer was carried out twice. The pellet was suspended completely during each washing. The pellet was briefly air dried (5-10 min) and 30 ul of H_2O was added in order to dissolve it at relatively high temperature of 55°C for 5 min. The samples were centrifuged at 14000rpm for 1 min. The supernatant was collected into new tube avoiding the contamination of silica powder. The eluted material was left at RT for 2-5 min and finally stored at -20°C.

2.4.5 Sequencing of the Gene

The purified sample was commercially sequenced by MACROGEN (Korea).

2.5.1 Analysis through Bioinformatics Tools

The cDNA sequence amplified through RT-PCR was subjected to different bioinformatics tools such as Justbio, Expasy, Peptide Property Calculator, kyte-doolittle entry form, PSIPRED.

2.5.2 Translation of nucleotide sequence into amino acid sequence

The nucleotide sequence was translated into their respective amino acids using Translator tool from Justbio (http://www.justbio.com/index.php?page=translator).

2.5.3 Determination of PI/Mw value

In order to compute Isoelectric Point (PI) and Molecular weight (Mw) of the enzyme, Compute pI/Mw tool was used (http://web.expasy.org/compute_pi/).

2.5.4 Determination of Physiochemical Properties

In order to determine different Physiochemical Properties such as Number of residues, Molecular weight, Extinction coefficient, Iso-electric point, water solubility and of the enzyme, Peptide Property Calculator was used (http://www.innovagen.com/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp).

2.5.5 Hydropathy Plot

Kyte-doolittle plot (http://gcat.davidson.edu/DGPB/kd/kd.cgi) was applied to determine the hydropathy of the enzyme.

2.5.6 Secondary structure of L asparaginase

The secondary structure for L asparaginase was predicted through PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/result/967646bc-e3db-11e3-858e-00163e110593).

2.5.6.1 Protein Secondary structure map

To find out the position of amino acids in helix, sheets, disorder regions and domain borders of the enzyme, secondary structure was constructed through PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/result/967646bc-e3db-11e3-858e-00163e110593).

2.5.7 Intrinsic disordered profile

The intrinsic disordered profile for the enzyme was determined through DISOPRED (http://bioinf.cs.ucl.ac.uk/psipred/result/967646bc-e3db-11e3-858e-00163e110593).

2.5.8 Gene Ontology terms prediction

For gene ontology terms prediction, FFPred (http://bioinf.cs.ucl.ac.uk/psipred/result/967646bc-e3db-11e3-858e-00163e110593), was used. Only biological and cellular functions were predicted through the software. The percentages of the individual amino acid in the enzyme were also determined by the software.

2.5.9 Alignment results

Alignments of the studied enzyme with other sources were determined through Gen THREADER (http://bioinf.cs.ucl.ac.uk/psipred/result/967646bc-e3db-11e3-858e00163e110593).

RESULTS

3.1 Genomic DNA Extraction

The DNA isolated from fruits and leaf parts of *Withania coagulans, Withania somnifera* and *Solanum lycopersicum* were assessed through 1% agarose gel as shown in Figure 3.1 and 3.2.The DNA extracted from fruit was comparatively of good quality as compared to those extracted from leaves.

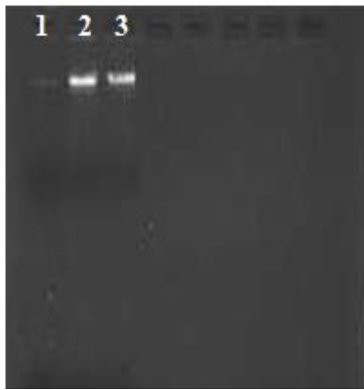

Fig 3.1:Extracted DNA from immature fruits

Lane 1 Withania coagulans
Lane 2: *Withania somnifera*
Lane 3: *Solanum lycopersicum*

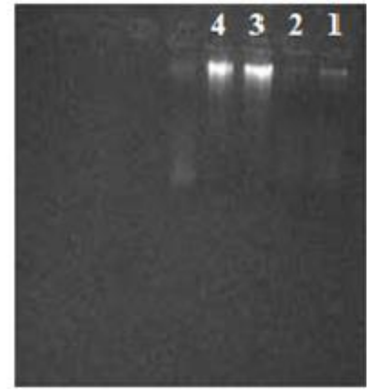

Fig 3.2: Extracted DNA from leaf

Lane 1: *Withania coagulans*
Lane 3: *Withania somnifera*
Lane 4: *Solanum lycopersicum*

3.1.1 PCR amplification of L asparaginase from Genomic DNA

Amplification of L asparaginase gene from Genomic DNA primers was not achieved so the gene was amplified from cDNA primers reported by (Oza et al., 2011).

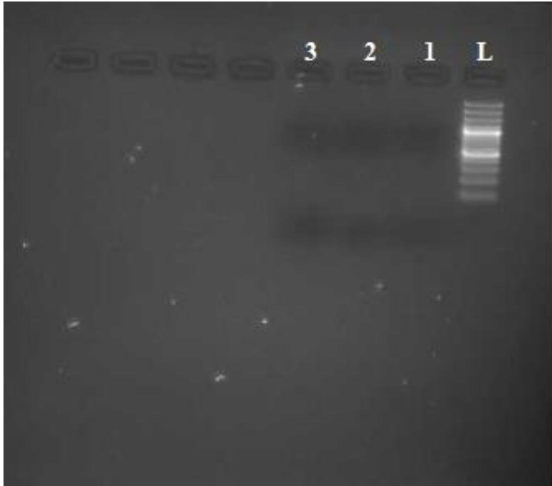

Fig 3.3: PCR amplification of L asparaginase gene from Genomic DNA

L: Ladder
Lane 1: *Withania coagulans*
Lane 2: *Withania somnifera*
Lane 3: *Solanum lycopersicum*

3.2: Total RNA

Since RNA is low molecular weight molecule than DNA, its quality was checked on 2% agarose. Three bands of RNA (i.e. mRNA, rRNA and tRNA) were observed in case of each sample. The RNA extracted from *Withania somnifera* (Fig. 3.5) was of good quality as compared to the rest of samples.

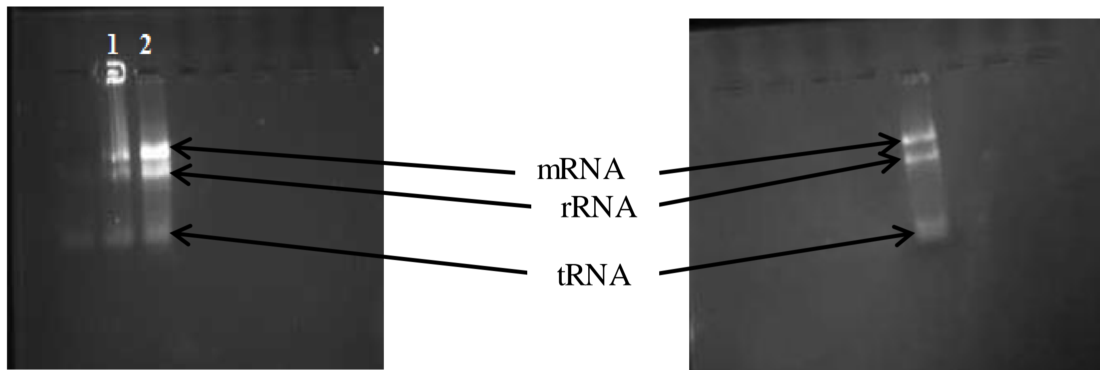

Fig 3.4: Extracted RNA from immature fruits **Fig 3.5: Extracted RNA from *W. somnifera***

Lane 1: *Withania coagulans*
Lane 2: *Solanum lycopersicum*

3.2.1 cDNA synthesis

For construction of cDNA libraries three types of primers (i.e Oligo dT18, Gene specific and random hexamers) can be used. Since random hexamer primers were used, smear was obtained in each case (Fig. 3.6) when run on 2% agarose gel. The smear depicted that whole of the transcriptome (i.e. mRNA, rRNA and tRNA) was reverse transcribed into cDNA.

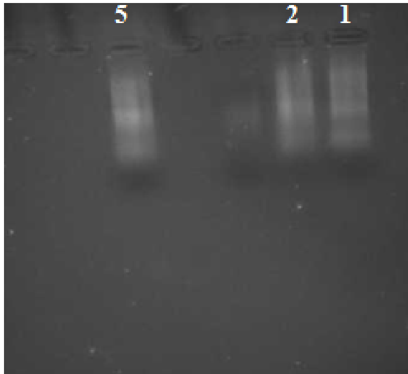

Fig 3.6: cDNA synthesis using random hexamers

Lane 1: *Withania coagulans*
Lane 2: *Withania somnifera*
Lane 5: *Solanum lycopersicum*

3.2.2 PCR amplification of L asparaginase gene from cDNA

L asparaginase gene of around 840 bp was amplified from cDNA of *Withania somnifera* but nonspecific bands were obtained as shown in Fig 3.7. So the band was excised using sterile blade and subjected to purification.

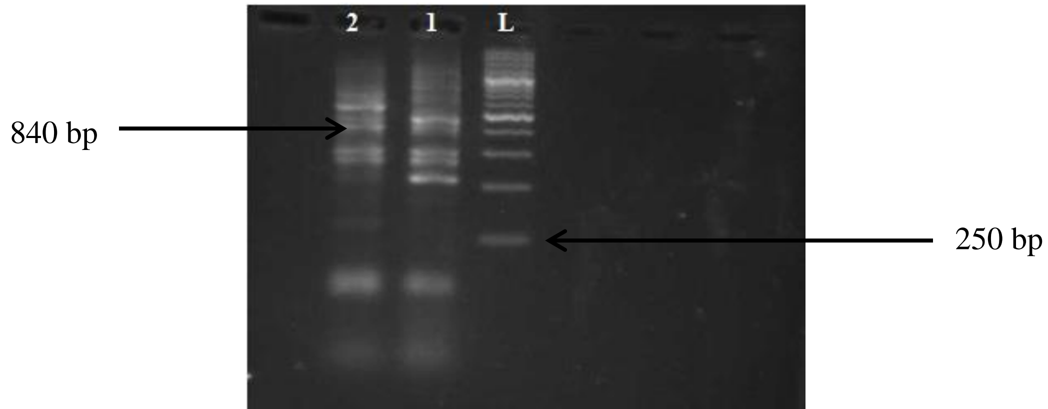

Fig 3.7: Amplification of L asparaginase gene from cDNA
L : Ladder ;
Lane 1 & Lane 2: *Withania somnifera*

The gel picture of the purified band of 840 bp is shown in Fig 3.8.

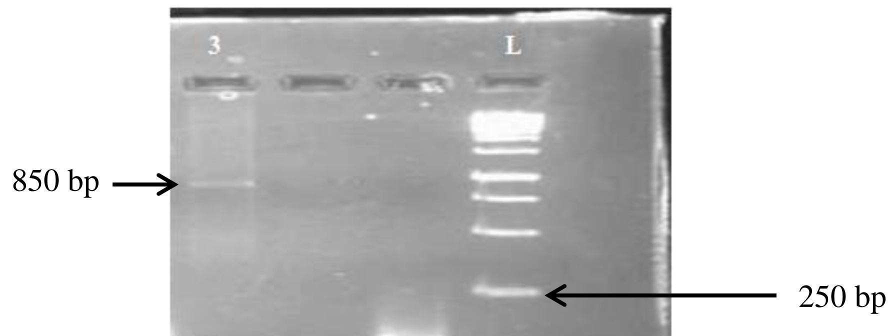

Fig. 3.8: Purified band of L asparaginase gene
L: Ladder, **Lane 3** Purified band

3.3.1 Analysis through Bioinformatics tools

3.3.1.1 Translation of nucleotide sequence into amino acid sequence

The Translate tool translated the cDNA sequence into amino acids sequence .The Translate tool showed L asparaginase enzyme with 280 amino acids with start codon AUG shown in appendix # 9 .Six Open Reading Frames (ORF) were identified by the software in which the biggest ORF had the length of 840 nucleotides (1-839) and the shortest one had length of 102(26-127) nuceotides. The forward primer (indicated by red colour) annealed within the 5' UTR and the reverse primer (indicated by green colour) at the 3' UTR region on the sequence obtained from National Centre for Biotechnology Information (NCBI)

3.3.1.2 Determination of PI/Mw value

The theoretical PI/Mw ratio of 5.06 / 29818.87 was obtained for the enzyme when computed through Compute pI/Mw tool. Since the PI value is directly related to the solubility of molecule, the enzyme has net charge of -11.5 at pH 7 which indicated that if water is used as a solvent for the enzyme it will easily solubilize in it.

3 .3.1.3 Physiochemical properties

The physiochemical properties of the enzyme from peptide property calculator have been summarized (Table 3.1). The peptide property calculator predicted that the enzyme can absorb maximum light at 12090 $M^{-1}cm^{-1}$.

Table 3.1 Physiochemical properties

S. No.	Properties	Value
1	Number of residues	280
2	Molecular weight	29818.97 g/mol
3	Extinction coefficient	12090 $M^{-1}cm^{-1}$
4	Iso-electric point	5.06
5	Net charge at pH 7	-11.5
6	Estimated solubility	Good water solubility

The Graph for Net Charge vs pH and Hydropathy are shown in Figure 3.9.

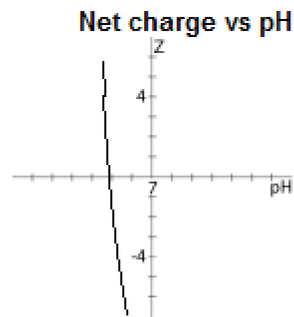

Fig 3.9: Net charge vs pH graph

3.3.1.4 Hydropathy plot

The Hydropathy Plot for the enzyme indicated that major portion of it lies in the Hydrophillic region as shown in Figure 3.10.

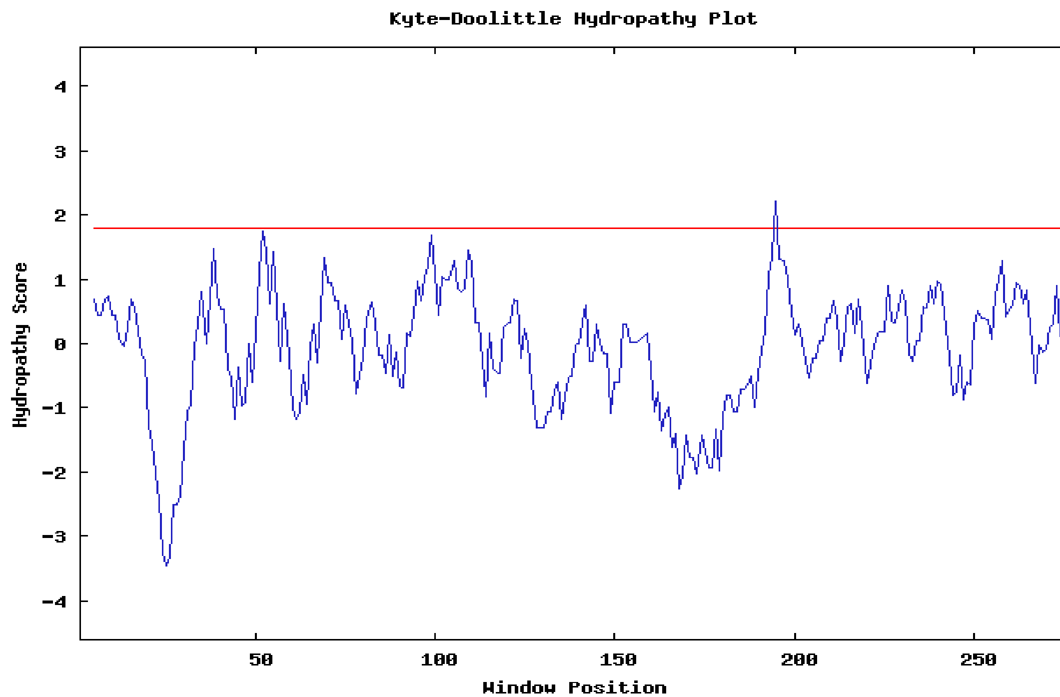

Fig 3.10: Hydropathy plot

3.3.1.5 Secondary structure of L asparaginase

The secondary structure for L asparaginase was predicted through PSIPRED and the structure showed that major portion of the enzyme was covered by helix and coil regions while very meager portion was in the form of strand (Fig 3.11). The Helix was indicated by cylindrical shapes that are coloured pink. The string portion of the enzyme was indicated by arrows that are coloured yellow while the coils are indicated by line that is coloured black.

In Figure 3.12 the amino acids that are present in the helix region of the enzyme are indicated by purple colour while those contributing to the sheet region are indicated by yellow colour. The amino acids that do not contribute to the tertiary structure of the enzyme are indicated in red colour. Due to the presence of helix and sheet regions, and absence of disordered protein binding regions the enzyme seems to be highly stable. The only amino acid that is present in the domain border region is Glycine which is highlighted dark blue.

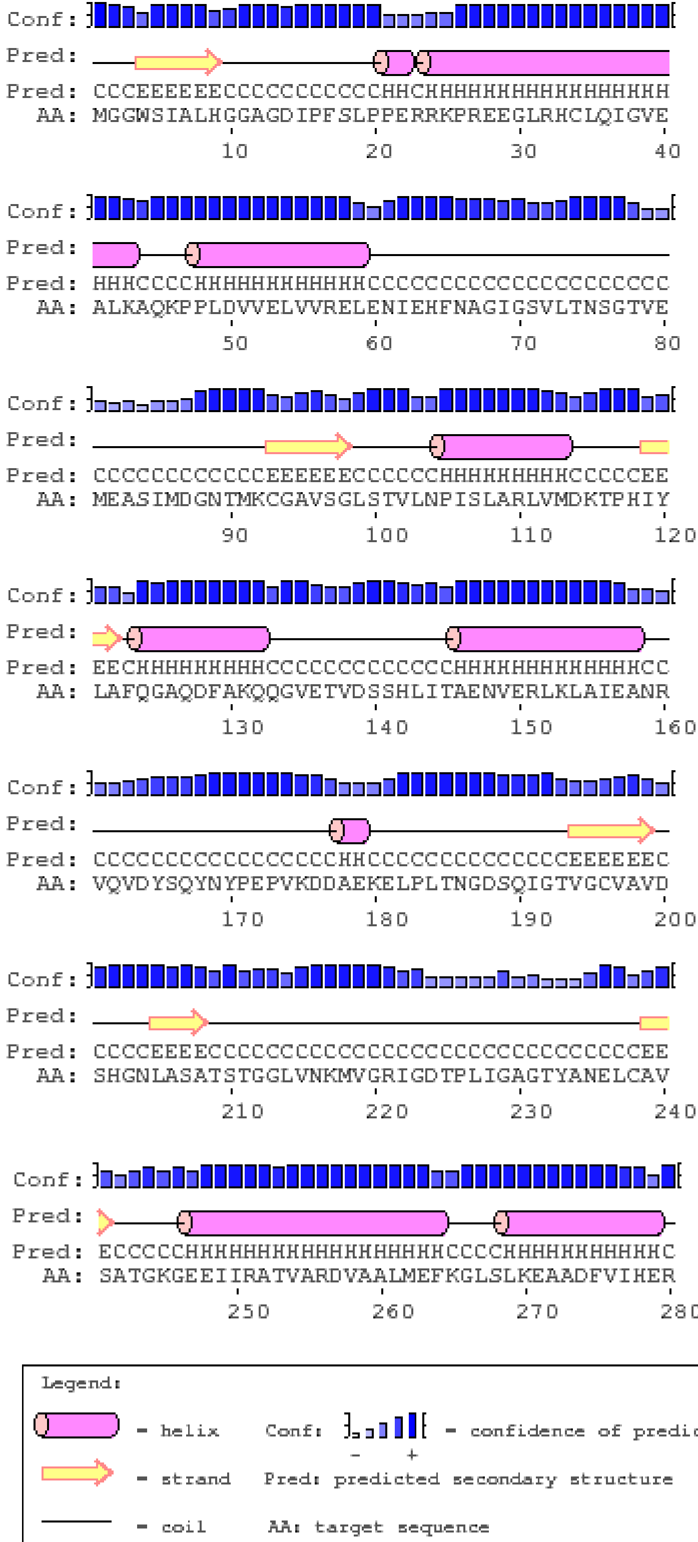

Fig 3.11: Secondary structure of L asparaginase

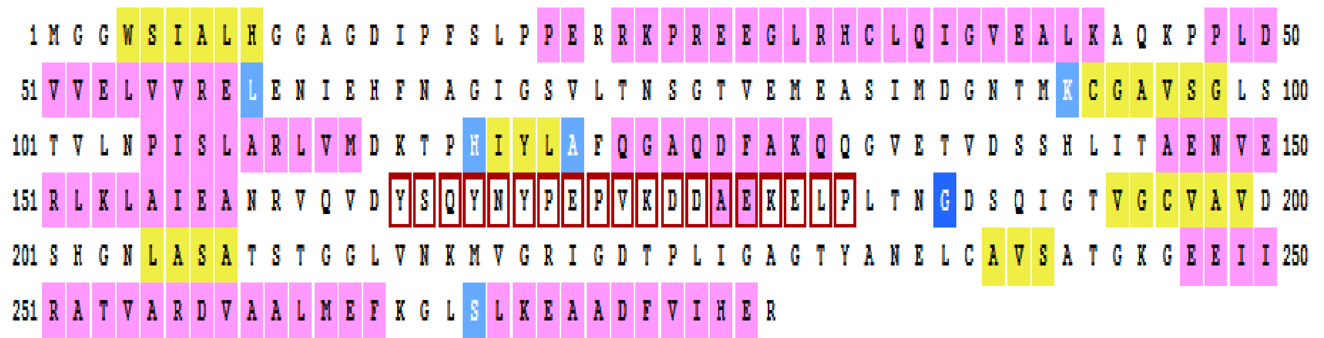

Fig 3.12: Secondary structure map of L-asparaginase

3.3.1.6 Intrinsic Disordered Profile

In the Figure 3.13, the blue line above the grey dashed line indicated that some amino acids are present in the disordered region. This means that if the graph goes above confidence score i.e. 0.5 the tertiary structure of the enzyme will go on disturbing. So for a most stable protein the graph should be below 0.5.The confidence of disordered protein binding residues predictions are indicated by orange lines. However as evident from the figure, the major portion of the amino acid sequence lies below 0.5 score so the enzyme is relatively stable.

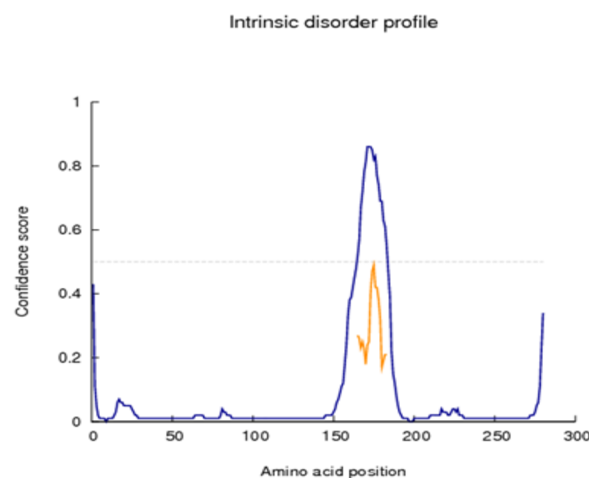

Fig 3.13: Intrinsic disordered profile

3.3.1.7 Gene Ontology terms prediction

Different biological functions such as role of the enzyme in phosphorylation of eukaryotic initiation factor 2α (GO:0016310), signal transduction (GO:0007165) and response of the enzyme to stimulus(GO:0051716) were predicted by the Gene ontology software. The software also predicted the role of the enzyme in regulation of transcription (GO:0006355) which was DNA dependent. The role of the enzyme in ATP binding (GO:0005524) and nucleotide binding (GO:0000166) was also predicted by the software which was categorized in molecular functions. The biological and molecular functions predicted by the software are shown in the Tables 3.2 and 3.3.

Table 3.2 Biological functions

S. No.	GO Term	Function
1	GO:0006796	phosphate-containing compound metabolic process
2	GO:0016310	phosphorylation
3	GO:0010468	regulation of gene expression
4	GO:0006355	regulation of transcription, DNA-dependent
5	GO:0009143	nucleoside triphosphate catabolic process
6	GO:0051716	cellular response to stimulus
7	GO:0006464	cellular protein modification process
8	GO:0007165	signal transduction
9	GO:0032502	developmental process
10	GO:0006950	response to stress
11	GO:0007275	multicellular organismal development
12	GO:0006325	chromatin organization

Table 3.3: Molecular functions

S. No.	GO Term	Function
1	GO:0005525	GTP binding
2	GO:0005524	ATP binding
3	GO:0000166	nucleotide binding
4	GO:0046872	metal ion binding
5	GO:0046983	Protein dimerization activity
6	GO:0042802	identical protein binding
7	GO:0019900	kinase binding

3.3.1.8 Amino acid Percentage

As evident from Table 3.4, among the Hydrophobic amino acid, Tryptophan is present at lowest percentage in the enzyme structure while glycine and alanine have the highest percentage i.e. 10% each. Among the hydrophilic amino acids, tyrosine is present at least percentage i.e. 1.79%. The software predicted the percentage of hydrophilic amino acids to be 20.72%.

Table 3.4 Amino acid percentage

Amino acid	**Symbol**	**Percentage (%)**
Alanine	A	10.00
Cysteine	C	1.43
Aspartic acid	D	5.00
Glutamic acid	E	8.21
Phenylalanine	F	2.14
Glycine	G	10.00
Histidine	H	2.50
Isoleucine	I	5.71
Lysine	K	4.64
Leucine	L	9.29
Methionine	M	2.50
Asparagine	N	4.29
Proline	P	4.29
Glutamine	Q	3.21
Arginine	R	4.29
Serine	S	6.07
Threonine	T	5.36
Valine	V	8.93
Tryptophan	W	0.36
Tyrosine	Y	1.79

3.3.1.9 Alignment results

The protein was aligned to different reported sources by GenTHREADER. Values for different parameters such as P-value, Pair-E, Solve-E etc. were determined. The total pairwise energy (Pair-E) was calculated. The pairwise energy of the enzyme aligned with Hydrolase (PDB id 3c17) was shown to be highest i.e. -552.9 and most favourable since the number of aligned amino acids was the highest i.e. 256 amino acids and the value indicated by negative sign for the protein in this case. As pairwise energy is directly related to the conformational state of enzyme so the conformational state of the enzyme in this case is highest. The pair wise energy of the protein with Hydrolase (PDB id 1apy) was the lowest and least favourable since 148 amino acids were matching with the sequence. Solvation Energy is related to the accessibility of individual atom of protein to the solvent. Solvation energy is also dependent on number of aligned amino acids of two proteins. It determines the stability of protein structures in water. In Table 3.5 the solvation energy of L asparaginase aligned with Hydrolase (PDB id 3c17) is -13.9 which means that the protein conformation is disturbed at very low expenditure of energy. Both pairwise energy and solvation energy determine the stability of protein conformation in water.

The alignment results obtained for L asparaginase with other reported sources are summarized in Table 3.5.

Table 3.5 Alignment results

S.No	PDB id	Name	Net Score	P Value	PairE	SolveE	Aln Score	Aln Len	Str Len	Seq Len
1	3c17	Hydrolase	126.85	8e-12	-552.9	-13.9	607.9	256	305	280
2	4o0c	Hydrolase	119.68	4e-11	-508.8	-16.1	568.6	241	296	280
3	4o47	Hydrolase	112.64	2e-10	-478.9	-10.0	534.7	240	295	280
4	2a8i	Hydrolase	109.24	5e-10	-523.4	-9.2	500	246	341	280
5	2gez	Hydrolase	106.97	8e-10	-269.8	-14.2	554.6	158	158	280
6	2a8j	Hydrolase	93.798	2e-08	-369.3	-4.3	439.0	228	310	280
7	3ljq	Hydrolase	90.302	4e-08	-381.9	-8.4	408.0	223	281	280
8	1k2x	Hydrolase	86.389	1e-07	-240.2	-11.3	423.4	153	156	280
9	1apy	Hydrolase	67.011	9e-06	-199.4	-5.0	307.0	148	161	280

Net Score: The GenTHREADER raw score
P-Value: Proportion of non-matching folds
Pair E: The Pairwise Energy
Solv E: The solvation Energy
Aln Score: The Pairwise alignment score
Aln Len: The length of the alignment

DISCUSSION

Much attention has been paid to L asparaginase in the past decade since it plays a key role in the treatment of acute lymphoblastic leukemia. The enzyme L asparaginase that is implied in clinical trials is derived from the microbial source (Narta et al., 2007). However, there are associated limitations with several adverse effects. These effects are probably due to the low Km value of the enzyme for Glutamine. To overcome the limitations, L asparaginase of plant origin was selected. Hence this study focused on finding an alternative plant source of L asparaginase by selecting three species of family Solanaceae.

There are fifty two species of family Solanaceae in Pakistan (Nasir and Ali, 1985) but the medicinal value of none has been elucidated at molecular level. In the present study three species: *Withania somnifera*, *W. coagulans* and *Solanum lycopersicum* has been selected. The choice of species was mainly based on the availability of fresh fruits needed for m*RNA* isolation. L asparaginase from *E. coli* and *E. crysenthemi* has been studied previously but little work has been reported on cloning and expression of L asparaginase from plant source. The cloning and expression of L asparaginase from *E. coli* and *E. carotovora* has been reported by Gilbert (1986) and Liu (1996) respectively.

Expression analysis: Procedures

Literature suggested the expression of genes in fresh fruis. However, the procedural details were missing. Therefore one of the challenge was to develop procedures for mRNA isolation from solanaceous plants. Previously such studies were not reported for *Withania* species. Therefore the legume model was used (Lough et al., 1992) for initial procedures of total RNA extraction followed by cDNA synthesis as reported in (Oza et al., 2011).

The reason for reporting *Withania somnifera* out of three species mentioned was successful implementation of protocol on this species, while these methods did not work with other two species. Hence the amplification only from cDNA of *Withania somnifera* has been reported with a product size of 840bp which revealed variation in the product size. Previously full length amplification was reported as 1000bp (Borek et al., 2004).

Sequence characterization and bioinformatics studies

The data obtained after sequencing was then subjected to different bioinformatics tools mentioned in materials and methods. The cDNA sequence was translated into its protein using Translate tool. The translate tool showed protein of 280 amino acids with methionine the starting amino acid and arginine the terminal one. This sequence matched 80.5% with that of *E. coli* L asparaginase previously reported (Jennings & Beacham, 1990, MAITA et al., 1979) with methionine the starting amino acid and tyrosine the terminal amino acid. When the enzyme was computed for its molecular weight, it was almost half of the molecular weight of L asparaginase reported for *E. coli* by (Cedar & Schwartz, 1967, Shifrin et al., 1974). The enzyme has net charge of -11.5 at pH 7 which showed that it has good solubility in water. This information was further strengthened by the pattern of Hydropathy plot which revealed the major portion of the enzyme in hydrophilic region. The intrinsic disordered profile showed that the enzyme was highly stable as major portion of the amino acids lye below the confidence score i.e. 0.5.

The biological functions such as role of the enzyme in response to stimulus were predicted by Gene ontology software (Pou et al., 1991). Moreover the software also predicted the probable function of the enzyme in phosphorylation as also previously been reported by (Mercado & Arenas, 1999). They also reported phosphorylation of the endogenous polypeptides in human cells. Role of the enzyme in response to stress was also predicted by the software, however the function is tissue specific (Reinert et al., 2006).

Among the alignment results determined for L asparaginase, three parameters i.e. Pair wise energies, solvation energies and number of aligned amino acid were taken into account as they are the factors that determine the conformational state of enzyme. The pairwise energy of the enzyme aligned with Hydrolase (PDB id 3c17) (Michalska et al., 2008) was the highest indicating the conformation of protein most favourable since the maximum number of amino acids aligned with the enzyme.

In summary, the study reports implementation of new protocols to study expression of a particular gene in *Withania somnifera*, an important medicinal plant in family solanaceae. The bioinformatics tools used to analyse the efficacy of L asparaginase revealed successfully

the structural and functional domains depicting its siginificant role in the treatment of Acute Lymphoblastis Leukemia.

CONCLUSIONS

Screening of selected members of family Solanaceae at molecular level revealed the occurrence of L. asparaginase in family Solanaceae. Protein secondary structure analysis showed the presence of helix, sheets, coils and disordered regions. The biological functions such as phosphorylation and regulation of gene expression assessed through bioinformatics tools signified the role of *Withania somnifera* L. asparaginase in treatment of Acute Lymphoblastic Leukemia.

FUTURE RECOMMENDATIONS

- How immunogenic the plant L asparaginase is? A question which needs to be explored.
- The efficacy of L. asparaginase in Acute Lymphoblastic Leukemia in clinical trials is another hard nut to crack
- The enzyme should be further characterized in the plants of family solanaceae.

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APPENDICES

Appendix # 1

CTAB Buffer 100ml

2.0g CTAB

10.0ml 1M Tris pH 8.0

4.0ml 0.5M EDTA pH 8.0

28.0ml 5M NaCl

40.0ml H_2O

Adjust all to pH 5.0 with HCL and make up a total of 100ml volume by adding distilled H_2O.

Appendix # 2

1 M Tris pH 8.0

With the help of magnetic stirrer dissolve 6.05g Tris base in 45ml of distilled water. Adjust pH to 8.0 by adding together the concentrated acid (HCL/H_2SO_4). Allow the solution, to cool 25°C before making the final adjustment to the pH. Adjust the volume to 50ml. Remember to autoclave it before use.

Appendix # 3

0.5M EDTA (pH 8.0)

7.306g EDTA

50ml Distilled water

Adjust pH 8.0 by NaOH crystal and autoclave before apply.

Appendix # 4

10 X TBE buffer

54 gm Tris base

27.5 gm boric acid

20ml of 0.5M EDTA (pH 8.0)

Make up to 500ml with water

Mix with magnetic stirrer and dissolve the chemical by adjusting the pH.

Appendix # 5

5M NaCl

14.625g NaCl

50ml Dist. H_2O

Mix with magnetic stirrer

Appendix # 6

T.E buffer

10 mM Tris-Cl, pH 8.0
1 mM EDTA
Make from 1M stock of Tris-Cl (pH 8.0) and 0.5M stock of EDTA (pH 8.0).
0.5ml 1M Tris-Cl pH 8.0 per 50ml
0.1ml 0.5M EDTA pH 8.0

Appendix # 7

Ethidium Bromide

Add 1 g of ethidium bromide to 100 ml of water. Stir on magnetic stirrer for several hours to ensure that the dye is dissolved. Wrap the container in aluminum foil or transfer the solution to dark bottle and store at room temperature (Sambrook *et al*., 1989).

Caution:

Ethidium bromide is a powerful mutagen and is moderately toxic. Gloves should be worn when working with solution that contain this dye and mask should be worn when weighing it out. After use, these solutions should be decontaminated.

Appendix # 8

6x Loading dye solution

10 mM Tris-HCl, pH 7.6
0.03% bromophenol blue
0.03% xylene cyanol FF
60% glycerol
60 mM EDTA

Storage:

Store at -20°C for best work (1year)

Appendix # 9

```
1 - ATGGGCGGCTGGAGCATTGCGCTGCATGGCGGCGCGGGCGATATTCCGTTTAGCCTGCCG - 60
     1 - M   G   G   W   S   I   A   L   H   G   G   A   G   D   I   P   F   S   L   P   -
20

    61 - CCGGAACGCCGCAAACCGCGCGAAGAAGGCCTGCGCCATTGCCTGCAGATTGGCGTGGAA -
120
    21 - P   E   R   R   K   P   R   E   E   G   L   R   H   C   L   Q   I   G   V   E   -
40

   121 - GCGCTGAAAGCGCAGAAACCGCCGCTGGATGTGGTGGAACTGGTGGTGCGCGAACTGGAA -
180
    41 - A   L   K   A   Q   K   P   P   L   D   V   V   E   L   V   V   R   E   L   E   -
60
```

```
   181 - AACATTGAACATTTTAACGCGGGCATTGGCAGCGTGCTGACCAACAGCGGCACCGTGGAA -
240
    61 - N  I  E  H  F  N  A  G  I  G  S  V  L  T  N  S  G  T  V  E   -
80

   241 - ATGGAAGCGAGCATTATGGATGGCAACACCATGAAATGCGGCGCGGTGAGCGGCCTGAGC -
300
    81 - M  E  A  S  I  M  D  G  N  T  M  K  C  G  A  V  S  G  L  S   -
100

   301 - ACCGTGCTGAACCCGATTAGCCTGGCGCGCCTGGTGATGGATAAAACCCCGCATATTTAT -
360
   101 - T  V  L  N  P  I  S  L  A  R  L  V  M  D  K  T  P  H  I  Y   -
120

   361 - CTGGCGTTTCAGGGCGCGCAGGATTTTGCGAAACAGCAGGGCGTGGAAACCGTGGATAGC -
420
   121 - L  A  F  Q  G  A  Q  D  F  A  K  Q  Q  G  V  E  T  V  D  S   -
140

   421 - AGCCATCTGATTACCGCGGAAAACGTGGAACGCCTGAAACTGGCGATTGAAGCGAACCGC -
480
   141 - S  H  L  I  T  A  E  N  V  E  R  L  K  L  A  I  E  A  N  R   -
160

   481 - GTGCAGGTGGATTATAGCCAGTATAACTATCCGGAACCGGTGAAAGATGATGCGGAAAAA -
540
   161 - V  Q  V  D  Y  S  Q  Y  N  Y  P  E  P  V  K  D  D  A  E  K   -
180

   541 - GAACTGCCGCTGACCAACGGCGATAGCCAGATTGGCACCGTGGGCTGCGTGGCGGTGGAT -
600
   181 - E  L  P  L  T  N  G  D  S  Q  I  G  T  V  G  C  V  A  V  D   -
```

```
200

   601 - AGCCATGGCAACCTGGCGAGCGCGACCAGCACCGGCGGCCTGGTGAACAAAATGGTGGGC -
660
   201 - S  H  G  N  L  A  S  A  T  S  T  G  G  L  V  N  K  M  V  G   -
220

   661 - CGCATTGGCGATACCCCGCTGATTGGCGCGGGCACCTATGCGAACGAACTGTGCGCGGTG -
720
   221 - R  I  G  D  T  P  L  I  G  A  G  T  Y  A  N  E  L  C  A  V   -
240

   721 - AGCGCGACCGGCAAAGGCGAAGAAATTATTCGCGCGACCGTGGCGCGCGATGTGGCGGCG -
780
   241 - S  A  T  G  K  G  E  E  I  I  R  A  T  V  A  R  D  V  A  A   -
260

   781 - CTGATGGAATTTAAAGGCCTGAGCCTGAAAGAAGCGGCGGATTTTGTGATTCATGAACGC -
840
   261 - L  M  E  F  K  G  L  S  L  K  E  A  A  D  F  V  I  H  E  R   -
280

   841 -  - 840
   281 - X                                          - 300
```

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