

## Review

# Seeking efficacy in L-asparaginase to combat acute lymphoblastic leukemia (ALL): A review

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L-asparaginase, a potential anti-leukemic drug, is used in clinics for the treatment of childhood acute lymphoblastic leukaemia, and is obtained from bacterial origin (*Escherichia coli* and *Erwinia carotovora*). The efficacy of L-asparaginase has remained under discussion for bearing many limitations, such as, early clearance from blood plasma and therefore requires multiple administration and various side effects causing immunogenic complications. The present article reviews studies that focused on seeking substitute in L-asparaginase by altering source, such as bacteria, fungi, actinomycetes, algae and plants, as well as reviews strategies like chemical modifications, protein engineering to overcome limitations in drug's efficacy.

**Key words:** L-asparaginase, acute lymphoblastic, leukemia, *Escherichia coli*.

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a blood cancer in which immature white blood cells or lymphocytes are enormously synthesized in bone marrow. Globally 3 in 100,000 individuals may acquire the disease mostly at an age under six years (Grigoriopoulos et al., 2013). However, chances of developing the disease are more at the age of 2 to 4 years.

So far, the exact pathogenic events leading to ALL have not been unveiled. The genetic syndromes (Down syndrome, Bloom's syndrome, ataxia telangiectasia and Nijmegen breakage syndrome), exposure to ultra frequency radiations and specific chemotherapeutic drugs may cause ALL (Hjalgrim et al., 2003). Parental occupation, maternal reproductive history, parental tobacco or alcohol use, maternal diet, prenatal vitamin use, exposure to pesticides or solvents and exposure to

the highest levels (>0.3 or 0.4 microteslas,  $\mu\text{T}$ ) of residential power-frequency magnetic fields, Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) in patients with mature B-cell could cause ALL (Bader, 1993; Lombardi et al., 1987).

Diagnosis of ALL starts with physical examination followed by complete blood count and blood smears using complete blood count (CBC) and blood smear tests. In most cases, blast cells are seen in the blood smears (Ramya et al., 2012b). Cytogenetics and immunotyping techniques can be used to detect whether the blast cells are derived from T cells or B cells. DNA testing can be used to find out the aggressiveness of the disease. CT scanning and ultrasound techniques can be used to find out the invasion of the disease in internal organs. In past steroids, chemotherapy, radiation therapy

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and combination therapy were introduced as ALL treatment.

The anti-proliferating properties of L-asparaginase on Leukemia cells were first identified and characterized in human clinical trials in 1970s. Since then, the enzyme has become a milestone in the treatment of ALL. Biochemically, it catalyzes the hydrolysis of 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 the non-acidic, hydrophilic amino acid both from blood serum as well as the self-synthesized amino acid in limited amount (Narta et al., 2007). L-asparaginase exploits the high demand of leukemia cells for asparagine by depleting the circulating pool of asparagine from the blood serum. Depriving them of L-Asparagine causes the death of cells. Deprivation of L-asparaginase amino acid causes death of the leukemia cells.

### Sources

The presence of L-asparaginase has been documented in a variety of organisms, such as animals, bacteria (Howard and Carpenter, 1972a), fungi (Sarquis et al., 2004), 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 in bacterial system such as *Escherichia coli* and *Erwinia carotovora* (Narta et al., 2007).

In order to use L-asparaginase as an epitome for the treatment of ALL, it should be devoid of glutaminase activity, high affinity for its substrate asparagine (low  $K_m$  value), no side effects, no immunogenic complications and delayed clearance from plasma (prolonged half-life) (Nagarethinam et al., 2012), so as to avoid frequent administration. L-asparaginase from bacterial source contain up to 10% L-glutaminase activity with no glutaminase activity for *Pyrococcus furiosus* L asparaginase (Campbell et al., 1967a; Bansal et al., 2010).

The glutaminase activity of L-asparaginase is probably due to structural identity in asparagine and glutamine. The structural formula suggested the similarity in amide groups, while glutamine maintains a difference of one additional methyl group (Ramya et al., 2012a). Similarly, during biosynthesis of asparagine, transamidation of aspartate occurs where glutamine serves as the amide group donor (Nagarethinam et al., 2012). The enzyme from *E. coli* and *E. carotovora* has gained worth in clinical applications against ALL, with  $K_m$  values of  $1.25 \times 10^{-5}$  M and  $1.8 \times 10^{-5}$  M, respectively (Cedar and Schwartz, 1967; Maita et al., 1974; Shifrin et al., 1974).

### Importance of L-asparaginase

The substrate of L-asparaginase, L-asparagine is from amide group amino acids and is of paramount nutrient

value for tumor cells. Due to enhanced rate of proliferation and metabolic processes taking place in cancerous cells, they require elevated level of amino acid (Luhana et al., 2013). The demand for asparagine is met in two ways: (a) from blood serum and (b) what they synthesize themselves. L-asparaginase as a drug that exploits this and hydrolyses L-asparagine into aspartic acid and ammonia thereby killing the tumor cells by depriving them from the amino acid (Narta et al., 2007). The low level of asparagine only affects the viability of cancerous cells without disturbing the normal cells. 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  $C_\gamma$  of the substrate asparagine forming a tetrahedral intermediate which then breaks down to form an acyl-enzyme intermediate. This is followed by the elimination of ammonia. A second nucleophile (normally water), then attacks this intermediate, thus transforming it to aspartic acid and free enzyme (Ehrman et al., 1971; Röhm and Van Etten, 1986) (Figure 1).

Asparagine has an important role in the biosynthesis of protein, DNA and RNA. Cells also need asparagine in order to proceed through G1 cycle in cell division (Rytting, 2012). The inability of leukemia cells to synthesize L-asparagine *de novo* is related to the lack or presence of low levels of asparagine synthetase (Kiriya et al., 1989; Prager and Bachynsky, 1968).

The L-asparaginase implied in clinics is from bacterial source, however, there are limitations or side effects for instance early clearance from blood plasma (short half-life), and immunogenic complications are associated with the drug.

### Limitations of L-asparaginase

Treatment of ALL with L-asparaginase is associated with side effects, such as leucopenia, neurological seizures, anaphylaxis, coagulation abnormalities, pancreatitis, etc. Another limitation is that the immune system of patients react against the drug in many different ways, such as depression of asparagine synthetase gene, production of specific antibodies against the drug, inactivation of caspase 3 or poly(ADP-ribose) polymerase (PARP) and production of glutamine in large quantities by adipocytes. Furthermore, the drug in its native form clears from blood serum (short half-life), so the patient needs 2 to 3 medications a week which results in frequent physician visit thereby making the overall treatment costly.

### Complications associated with L-asparaginase therapy

The 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; Viganò-D'Angelo et al., 1990; Trivedi and Pitchumoni, 2005) and anti-thrombin III (Aoki et al., 2005; Brodtman et al., 2005; Sahoo and Hart, 2003b). Development of acute pancreatitis is also one of the side effects and a well-documented complication 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 leukaemia therapy (Haddy et al., 2006; Hernandez-Espinosa et al., 2006). During therapy, the onset of venous thrombosis in children has been reported (Sahoo and Hart, 2003a). In adolescent patients with leukaemia develop cerebral thrombotic complications due to L-asparaginase therapy (Mitchell et al., 1994). Growth hormone deficiency, particularly, in children (Omoti and 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).

## Toxicity

L-asparaginases were found to be associated with corneal toxicity in patients given combinational chemotherapy. Foreign body sensation, blur red vision, ocular pain, and bilateral conjunctival hyperaemia were found to be the common symptoms observed (Sutow et al., 1971). Due to L-asparaginase therapy, myocardial ischemia has been reported in patients with ALL. 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 and 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 (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 central nervous system (CNS) functions along with agitation, hallucinations, disorientation, convulsions and coma were observed (Korholz et al., 1987). Korholz et al. (1987) also 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 were due to the production of high titres of Immunoglobulin G3 (IgG3) antibodies associated to a higher risk of anaphylaxis. Many reports clearly indicate that *E. coli* L-asparaginase causes more hypersensitive reactions when compared with *Erwinia* species.

## Immunogenic complications/resistance to the drug

Different researchers have presented different opinions about resistance to L-asparaginase. Ho et al. (1970) and Worton et al. (1991) both have reported that resistance to the drug is primarily due to depression of asparagine synthetase gene. The resistance has been related to the production of specific antibodies against the drug (Capizzi, 1993). The cells sensitive to L-asparaginase produce cytokines (Gallagher et al., 1989). These cytokines control the expansion of resistant cells. As soon as the sensitive cells are killed by L-asparaginase, the resistant cells escape from regulatory control. The cytokines (IL 12 family members) have direct anti-tumor activity against B-ALL (Cocco and Airoldi, 2011). It has also been reported that the cytokines, G-CSF and GM-CSF activate quiescent leukaemia cells, stimulate their proliferation and make them sensitive to chemotherapeutic drugs. However, Kullas et al. (2012) reported that L-asparaginase suppresses the production of cytokines. Holleman et al. (2003) associated the resistance to L-asparaginase and prednisolone to inactivation of caspase 3 or PARP (Holleman et al., 2003). Ehsanipour et al. (2013) have established a link of obesity with L-asparaginase resistance and documented that adipocytes (fat cells) which produce sufficient quantities of glutamine cause the leukaemia cells to resist against L-asparaginase (Ehsanipour et al., 2013). So their findings suggest that obese children have high risk of leukaemia compared to their lean counterparts.

## Strategies to overcome limitations

To overcome these limitations, several strategies have been applied which include chemical modification (Yoshimoto et al., 1986), protein engineering (Offman et al., 2011) by site directed mutagenesis (Mehta et al., 2014a) and searching for novel source of the drug.

## Chemical modifications

Three preparations, that is, two native and one modified

**Table 1.** Comparison of native and modified L-asparaginase.

Parameter	<i>E. coli</i>		<i>Erwinia</i> (Native)
	Native	PEGylated	
Activity(IU)/mg protein	280-400	280-400	650-700
$K_m$ ( $\mu$ M)-l-asparaginase	12	12	12
$K_m$ ( $\mu$ M)-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

form (from either of the native forms) of L-asparaginase used in clinics are available. The native forms of the enzyme have been isolated from *E. caratovora* and *E. coli*. The molecular weight of the enzyme isolated and purified from various strains of *E. coli* range from 132 to 141 kDa (Irion and Arens, 1970; Jackson and Handschumacher, 1970; Maita and Matsuda, 1980) while the molecular weight of *Erwinia* L-asparaginase turns out to be 138 kDa. The purified enzyme has specific activity that lies between 300 and 400  $\mu$ mol 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 and Carpenter, 1972b). The approximate  $K_m$  value for L-asparaginase is  $1 \times 10^{-5}$  mol/L (Jackson and Handschumacher, 1970; Maita and Matsuda, 1980). These asparaginases contain up to 10% glutaminase activity which is undesirable.

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 the 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 polyethylene glycol [PEG]) has been the most reliable method applied to the drug. Later, the enzyme was successfully conjugated to PEG (Abuchowski et al., 1979).

The anti-leukemic properties of this modified 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 subjected to freeze thaw 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, that is, biweekly as opposed to 2 to 3 medications a week of native *E. coli* preparation (Narta et al., 2007). The actual cost of PEG-asparaginase is greater than that of native forms, 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., 2007). PEG L-asparaginase is commercially available as PEG-asparaginase by generic name, and ONCASPAR is the trademark of the manufacturer, ENZON, South Plainfield, NJ. Comparison of native and modified L-asparaginases is as shown in Table 1.

Several other chemical modifications of the enzyme with their outcomes are summarized in Table 2. The aforementioned modifications are those made to bacterial L-asparaginase, however, Karamitros et al. (2013) have encapsulated poorly characterized yeast asparaginase (ScASNase) with two or three layers poly dextran/poly-L-arginine-based bilayers. As an outcome improved resistance against proteases such as trypsin and thrombin and thermal inactivation at 37°C were observed. This could significantly extend its half life *in vivo* during its implementation in ALL. Enzyme from *Cladosporium* species (fungi), has also been subjected to chemical modification with bovine serum albumin, ovalbumin by cross-linking using glutaraldehyde, N-bromosuccinimide, and mono-methoxy polyethylene glycol (Mohan et al., 2014). Modification with ovalbumin resulted in improved enzyme activity that was 10-fold higher compared to native enzyme, while modification with bovine serum albumin through glutaraldehyde cross-linking resulted in high stability of L-asparaginase that was 8.5 and 7.62 fold more compared to native enzyme at 28 and 37°C by the end of 24 h. Modification also markedly prolonged L-asparaginase half-life and serum stability. N-Bromosuccinimide-modified ASNase presented greater stability with prolonged *in vitro* half-life of 144 h to proteolytic digestion relative to unmodified enzyme (93 h), but far more than native *E. coli* form (20 h). But *in vitro* trials for associated side effects and percent glutaminase activity for the modified form on human and animal model

**Table 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 polyoxyethyleneallyl methyl diether	Immunoreactivity towards anti-asparaginase serum was lost completely (Kodera et al., 1992)
Silk fibroin-L-ASNasebioconjugate	Lower immunogenicity and antigenicity compared with the native enzyme (Zhang et al., 2005) and has increased thermal stability (Wang and Cao, 2011)

are still awaited.

### Protein engineering

Several attempts have been made at protein level to produce L-asparaginase that would be glutaminase free, possessing no side effects and prolonged half-life so as to ensure successful implementation of *in vivo* treatment of ALL. So the purpose of engineering L-asparaginase should be to identify the amino acid residues that play a central role in contributing to enzyme stability, specificity and immunogenicity. Aghaiypour et al. (2001) presented high-resolution crystal structures of the complexes of *Erwinia chrysanthemi* L-asparaginase (ErA) and by comparing the amino acid sequence and crystal structure of ErA with other bacterial sources, found that replacement of glutamine and asparagine amino acids with active sites residues, Glu63 and Ser254 may decrease the glutaminase activity of L-asparaginase (Aghaiypour et al., 2001).

Gaofu et al. (2005) constructed a recombinant gene encoding a chimeric protein comprising of asparaginase, a tetanus toxin peptide (TTP)

spacer (831–854 fragment), and the foreign cholesteryl ester transfer protein C-terminal fragment (CETPC). The gene was expressed and directed to the periplasmic region of *E. coli* and the purified chimeric enzyme showed approximately 83% activity to that of the native (Gaofu et al., 2005).

Jianhua et al. (2006) applied alanine-scanning mutagenesis to L-asparaginase from *E. coli* the purpose of which was to point out the residues responsible for the enzyme antigenicity (Jianhua et al., 2006). Four mutant recombinant L-ASPs were constructed and expressed in *E. coli*, and then purified. The change of alkaline residues from 195RKH 197 to 195AAA 197 significantly declined the antigenicity of enzyme assessed through competition enzyme-linked immunosorbent assay using polyclonal antibodies raised against the wild-type L-ASP from rabbits.

Kotzia and Labrou (2009) used *in vitro* directed evolution to create a new enzyme variant with better thermal stability. Using the genes encoding L-asparaginases from *E. chrysanthemi* and *E. Carotovora*, a library of enzyme variants was generated by a staggered extension process. The parental L-asparaginase amino acid sequences revealed 77% identity, but their half-inactivation

temperature ( $T_m$ ) varies by 10°C. A thermostable variant of the *E. chrysanthemi* enzyme was identified to contain a single point mutation (Asp133Val). The  $T_m$  of this variant was 55.8°C, whereas the wild-type enzyme has a  $T_m$  of 46.4°C. At 50°C, the half-life values for the wild-type and mutant enzymes were 2.7 and 159.7 h, respectively. Analysis of the electrostatic potential of the wild-type enzyme revealed that Asp133 is located at a neutral region on the enzyme surface and makes a significant and unfavourable electrostatic contribution to the overall stability. The contribution of position 133 to thermostability of the enzyme was further analysed through site-saturation mutagenesis. A library of random Asp133 mutants was screened out which confirmed the involvement of this position in thermostability and showed that the Asp133Leu mutation confers optimal thermostability (Kotzia and Labrou, 2009).

Offman et al. (2011) successfully engineered *E. coli* L-asparaginase which resisted against degradation and inactivation by 3-amino enolpyruvate 2-phosphate(AEP) produced by leukemic blast cells. Two mutants N24A and N24A R195S were designed. The reduced dosage of the former resulted in reduced antigenicity

while the latter exhibited reduced glutaminase activity and toxicity. So these two mutants could be exchanged during cancer therapy according to the needs and observed side effects of the patient (Offman et al., 2011).

Mehta et al. (2014) performed rational protein engineering approach and made several mutations in L-asparaginase-II of *E. coli* to find out the amino acid residues that contribute to enzyme stability, specificity towards asparagine and glutamine, immunogenicity and ability of killing leukemia cells (Mehta et al., 2014b). *E. coli* L-asparaginase-II variants with double mutation K288S/Y176F (that is, replacement of Lys288 with Serine and Tyr176 with Phenylalanine) were considerably less recognized by serum antibodies from mice immunized with wild-type L-asparaginase-II and an ALL patient who had been given asparaginase therapy for 45 days. In addition, such variants were also significantly stable as compared to wild type of the enzyme. Except for mutation Y176S, that is, replacement of tyrosine (Y) with serine (S), all other mutations made at position 176 significantly reduced glutaminase activity of the enzyme without disturbing its asparaginase activity. So the replacement of tyr176 with Phe and double mutation K288S/Y176F caused significant reduction in glutaminase activity, however, had somewhat higher asparaginase activity and significantly enhanced cytotoxicity as compared with wild-type *E. coli* L-asparaginase. This double mutated variant exhibited reduced antigenicity and was 10 fold less immunogenic as compared to wild type. The variant with residue Y176 for which a mutation to S, became devoid of glutaminase activity but had significantly reduced cytotoxicity against leukemia cells as compared to the wild type of enzyme.

Interestingly, substitutions made at positions 176 (Y176F) and 66 (W66Y) resulted in variants with elevated glutaminase activity, but higher cytotoxicity against leukemia cells. This provided a new insight that in spite of its glutaminase activity, the therapeutic efficiency of the enzyme can be improved through such mutations. So L-asparaginase variants possessing lower glutaminase activity, but high cytotoxicity, also appears to be effective drugs in future. Chan et al. (2014) applied saturation mutagenesis to *E. coli* L-asparaginase followed by enzymatic screening and identified Q59L (replacement of glutamine with leucine) variant as a promising one that retained asparaginase activity without exhibiting any detectable glutaminase activity (Chan et al., 2014). So their observation offers a striking rationale for further consideration of Q59L as a drug candidate.

### Searching for novel sources of L-asparaginase

On the basis of amino acid sequence and biochemical properties, enzyme possessing asparaginase activity can be categorized into several families (Borek and 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 important in cancer therapy have been discussed.

### Bacterial L-asparaginase

In bacteria, two types of L-asparaginase, that is, Type 1 and Type II have been reported (Campbell et al., 1967b). Type 1 are expressed in cytoplasm and hydrolysed both in 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-asparagine which is the main nutrient for tumor cells. The presence of L-asparaginase has been reported from many bacteria, such as *E. coli*, *E. carotovora*, *Bacillus* species, *Pseudomonas* species, 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-asparaginases with their biochemical properties are summarized in Table 3.

Cappelletti et al. (2008) reported a new L-asparaginase from *Helicobacter pylori*. Enzyme from this source has the lowest glutaminase activity, that is, 0.01% and require optimum pH of 7 to 10 for its asparaginase activity. But as evident from Table 3, its  $K_m$  value is relatively higher than *E. coli* L-asparaginase and its higher cytotoxic effects were also reported. Recombinant ASNase derived from *Wolinella 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 needs 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  $K_m$  value (that is,  $2.2 \times 10^{-5}$  M) for glutamine which is undesirable and hence this character would probably limit its application in cancer therapy.

Angelica et al. (2012) have reported *Rhizobium etli* as another novel source of L-asparaginase. On the basis of glutaminase free activity and biochemical properties determined, the enzyme from this source is proposed to be a potential drug for the treatment of ALL. So further studies are needed to authenticate its use as an anticancer therapeutic drug.

### Fungi L-asparaginase

L-asparaginase is also efficiently produced by fungi and the enzyme from fungal source have less side effects in comparison to the bacteria (Sarquis et al., 2004). The production of L-asparaginase by filamentous fungi, namely, *Aspergillus tamarii* and *Aspergillus terreus* have

**Table 3.** Biochemical properties of some bacterial asparaginases.

Bacteria	Biochemical properties				
	Opt pH	Opt.Temp (°C)	$K_m$ (M) L-asparagine	MW (kDa)	References
<i>Pseudomonas aeruginosa</i>	9	37	$0.147 \times 10^{-3}$	160	El-Bessoumy et al. (2004)
<i>Proteus vulgaris</i>	7-8	57	$2.6 \times 10^{-5}$	-	Tosa et al. (1972)
<i>Vibrio succinogenes</i>	7.3	-	$4.78 \times 10^{-5}$	146	Distasio et al. (1976)
<i>Bacillus coagulans</i>	8.5-9.5	55	$4.7 \times 10^{-3}$	85	Law and Wriston (1971)
<i>Mycobacterium phlei</i>	8.8-9.2	-	$0.7 \times 10^{-3}$	126	Rozalska (1989)
<i>Erwinia carotovora</i>	8	50	$1.8 \times 10^{-5}$	125-145	Maladkar et al. (1993), Cammack et al. (1972)
<i>Erwinia aroideae</i>	7.5	-	$3 \times 10^{-3}$	155	Peterson and Ciegler (1969), Tiwari and Dua (1996)
<i>Escherichia coli</i>	7-8	37	$1.25 \times 10^{-5}$	141	Cedar and Schwartz (1967), Shifrin et al. (1974)
<i>Azotobacter vinelandii</i>	8.6	48	$1.1 \times 10^{-4}$	84	Gaffar and Shethna (1977)
<i>Pseudomonas fluorescens</i>	8-9	-	$4.1 \times 10^{-4}$	70	Nilolaev et al. (1975)
<i>Pseudomonas stutzeri</i>	9	37	$1.45 \times 10^{-4}$	34	Manna et al. (1995)
<i>Pseudomonas aeruginosa</i>	9	37	$0.147 \times 10^{-3}$	160	El-Bessoumy et al. (2004)
<i>Acinetobacter calcoaceticus</i>	8.6	-	$2 \times 10^{-3}$	105	Joner et al. (1973)
<i>Bacillus subtilis</i> B11-06	7.5	40	$4.3 \times 10^{-4}$	38 each subunit	Rao et al. (2013)
<i>Corynebacterium glutamicum</i>	7	40	$2.5 \times 10^{-3}$	81	Mesas et al. (1990)
<i>Helicobacter pylori</i>	7-10	45	$2.9 \times 10^{-4}$	140	Cappelletti et al. (2008)
<i>Aeromonas</i> spp.	9	50	$4.9 \times 10^{-6}$	-	Pattnaik et al. (2000)
<i>Pyrococcus furiosus</i>	9	-	$1.2 \times 10^{-4}$	-	Bansal et al. (2010)
<i>Rhizobium etli</i>	9	37	$8.9 \times 10^{-3}$	102	Angelica et al. (2012)
<i>Photobacterium</i> spp. strain J15	7	25	$7.6 \times 10^{-4}$	70	Yaacob et al. (2014)
<i>Bacillus licheniformis</i>	6-10	40	$1.4 \times 10^{-5}$	134.8	Mahajan et al. (2014)
<i>Bacillus aryabhatai</i>	8.5	40	$2.57 \times 10^{-4}$	155	Singh et al. (2013)

been reported to produce L-asparaginase with the highest production in 2% proline medium from *A. terreus* (Sarquis et al., 2004). Ali et al. (1994) have reported that *A. terreus* 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 cerevisiae* and is encoded by the ASP3 gene (Bon et al., 1997). The production of L-asparaginase has also been reported from *Pichia polymorpha*, to be isolated

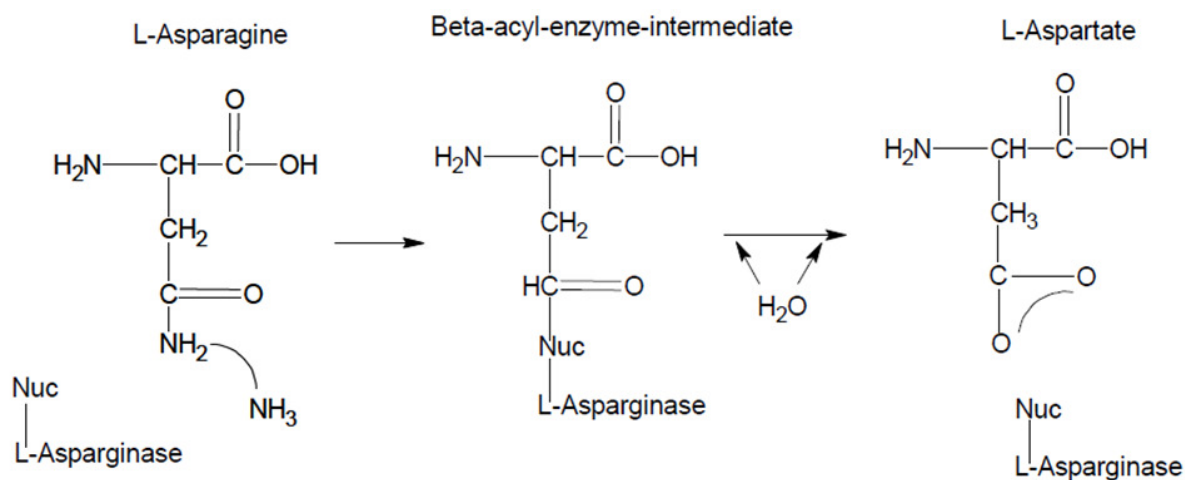
from Egyptian soils by enrichment method (Foda et al., 1980). The enzyme has also been isolated from the cell culture broth of *Candida utilis* (Kil et al., 1995). The enzyme from *C. utilis* has low  $K_m$  value, but it has less affinity towards L-asparagine and clears from plasma at faster rate indicating 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 the presence of mannan moiety in it. Some of the biochemical properties of fungi L-

asparaginase are summarized in Table 4.

Recently, a marine fungus *Beauveria bassiana* (MSS18/41) has been reported to produce L-asparaginase (Nageswara et al., 2014), but it has not been so far various characterized parameters considered in ALL treatment. The enzyme from *Mucor hiemalis* possesses desirable traits such as stability at physiological pH and temperature, high substrate specificity and good scavenging activity. So, the enzyme need to be explored as an antileukemic agent.

**Table 4.** Biochemical properties of fungi L-asparaginase.

Fungi	Biochemical properties				
	Opt pH	Opt Temp (°C)	$K_m$ (M)	MW (kDa)	Reference
<i>Saccharomyces cerevisiae</i>	6.8	-	$3.5 \times 10^{-4}$	800	Dunlop et al. (1978)
<i>Pichia polymorpha</i>	6.7	-	$1.37 \times 10^{-2}$	-	Foda et al. (1980)
<i>Rhizomucor miehei</i>	7	45	-	133.7	Huang et al. (2013)
<i>Penicillium digitatum</i>	7	30	$1 \times 10^{-5}$	-	Shrivastava et al. (2012)
<i>Candida utilis</i>	-	-	$7.7 \times 10^{-5}$	480	Sakamoto et al. (1977)
<i>Penicillium</i> spp.	7	37	$4 \times 10^{-3}$	66	Patro and Gupta (2012)
<i>Aspergillus aceulatus</i>	9	30	$12.5 \times 10^{-3}$	-	Dange and Peshwe (2011)
<i>Fusarium tricinctum</i>	7.5-8.7	-	$5.2 \times 10^{-4}$	161-170	Scheetz et al. (1971)
<i>Aspergillus terreus</i>	5-7	40-45	$5.8 \times 10^{-4}$	-	Aliet al. (1994)
<i>Cylindrocarpon obtusisporum</i>	7.4	37	$1 \times 10^{-3}$	216	Raha et al. (1990)
<i>Cladosporium</i> spp.	6.3	30	$1 \times 10^{-1}$	121	Kumar and Manonmani (2013)
<i>Aspergillus niger</i>	6	37	$1.6 \times 10^{-5}$	-	Luhana et al. (2013)
<i>Mucor hiemalis</i>	7	37	$4.3 \times 10^{-3}$	96	Monica et al. (2013)

**Figure 1.** Asparagine degradation by L-asparaginase.

The enzyme has also been sufficiently characterized in fungi so it should be processed ahead towards clinical trials.

### 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 to 55% are produced by this genus (Subramani and 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 *Streptomyces karnatakensis*, *Streptomyces venezualae*, *Streptomyces longisporusflavus*, and *Streptomyces ginsengisoli* (Deshpande et al., 2014) and a marine *Streptomyces* spp. PDK2 have been reported to produce L-asparaginase (Mostafa and Salama, 1979; Mostafa, 1979; Abdel et al., 1998; Dhevagi and Poorani, 2006). There are also reports of L-asparaginase production from some *Streptomyces* spp. isolated from the gut of the fish *Therapon jarbua* and *Villorita cyprinoides*, *Streptomyces*



**Table 5.** Biochemical properties of plant L-asparaginase.

Plant	Biochemical properties				
	pH	Temp (°C)	$K_m$ (M)	MW (kDa)	Reference
<i>Withania somnifera</i>	8.5	37	$6.1 \times 10^{-5}$	72	Oza et al. (2009a)
<i>Capsicum annum</i>	8.5	37	$3.3 \times 10^{-3}$	120	Bano and Sivaramakrishnan (1980)
<i>Pisum sativum</i>	-	-	$2.4 \times 10^{-3}$	69	Chagas and Sodek (2001)
<i>Lupinus arabeus</i>	-	-	$6.6 \times 10^{-3}$	75	Chang and Farnden (1981)
<i>Lupinus angustifolius</i>	-	-	$7 \times 10^{-3}$	75	Chang and Farnden (1981)

*gulbargensis* and *Streptomyces noursei* MTCC 10469 (Dharmaraj, 2011; Amena et al., 2010). Gupta et al. (2007) reported marine actinomycetes to be a potential source of L-asparaginase, for they have extreme adaptation within marine environment. L-asparaginase purified and characterized from *Streptomyces* phosphotransferase gene (sph) as the  $K_m$  value of  $2.5 \times 10^{-5}$  M with optimum pH of 7.5 for enzyme activity which is very close to pH of human blood, but the enzyme shows optimum activity at 50°C which is far higher than human body temperature (Basha et al., 2009). Han et al. (2014) have characterized the enzyme isolated from *Staphylococcus* species. OJ82 with optimum temperature, pH and  $K_m$  value of 37°C, 9 and  $2.2 \times 10^{-3}$  M, respectively. 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 properties, such as optimum pH, optimum temperature for activity, percent glutaminase activity, toxicity and  $K_m$  value of the enzyme for its substrate L-asparagine from actinomycetes sources.

### Algae L-asparaginase

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

### Plant L-asparaginase

L-asparaginase has been reported from a number of plant species, such as Barley roots (Grover and Chibnall, 1927), seedling of *Lupinus leutus* and *Dilichos lab* (Lees and Blakeney, 1970), Green chilli (Bano and Sivaramakrishnan, 1980), *Tamarindus indica* (Bano and Sivaramakrishnan, 1980), *Lupinus angustifolius* (Dickson et al., 1992), soyabean leaves (Cho et al., 2007), leaves,

flowers buds and root tips of *Lupines arabeus* (Lough et al., 1992b), *Arabidopsis thaliana* (Bruneau et al., 2006), cotyledons of chickpea seedlings (El-Shora and Ali, 2013), seeds of *Vicia faba* and *Phaseolus vulgaris* (Sanaa et al., 2012), immature seeds of pea (Sodek et al., 1980), *Lotus japonicas* (Credali et al., 2011), *Withania somnifera*, *Datura innoxia*, *Lycopersicon lycopersicum*, *Vigna unguiculata*, *Asparagus officinalis*, *Oryza sativa* (Oza et al., 2009a). L-asparaginase activity has also been reported in soil of the roots of *Pinus pinaster* and *Pinus radiata* (Bell and 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 glycosylasparaginase (Lough et al., 1992a; Lough et al., 1992b). The biochemical properties of L-asparaginase from some of the plant sources are summarized in Table 5.

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 *W. 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  $K_m$  value (for asparagine) of the enzyme cloned and expressed in *E. coli* from this source came out to be  $7.02 \times 10^{-5}$  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. El-Sayed et al. (2012) investigated the antitumor activity of the enzyme from *Vicia faba* and *Phaseolus vulgaris* on human cell lines hepatocellular carcinoma (Hep-G2). The optimum pH for the enzyme activity at 37°C from both sources was 7.5 to 8.5 for immobilized one while a pH of 7.0 for free enzyme.

## CONCLUSIONS

The forgone discussion reveals the importance of L-asparaginase in ALL treatment. Literature suggests that side effects of the enzyme are probably due to its glutaminase side activity. However there are some other requirements for the enzyme unfulfilment which could be the cause for serious side effects. One of the requirements is prevailing pH environment of human blood. As the pH of human blood is around 7.4, so we need the enzyme which would perform efficiently under such pH conditions. Another requirement is the human body temperature which is 37°C. So source of the enzyme with maximum activity at this temperature would be preferred in this case.

As L-asparaginase converts asparagine to aspartic acid and ammonia, this would definitely enhance the concentration of aspartic acid in blood serum. So due to acidic nature of aspartate, the pH of the blood serum will become lowered. This disturbance in the blood pH might have been the unexplored cause of the side effects. So to neutralize the effect of aspartic acid would be an extremely hard nut to crack.

Mehta et al. (2014) reported that when substitutions of amino acids at positions 176 (Y176F) and 66 (W66Y) were made to the enzyme sequence, the enzyme acquired lower glutaminase activity but higher cytotoxicity against cancer cell lines. So L-asparaginase possessing lower glutaminase activity but higher cytotoxicity may appear to be an effective drug in future.

L-asparaginase subjected to extensive mutations is from the bacterial origin. It has been identified in other sources, to make mutations in the enzyme sequence from source other than bacteria.

## Conflict of Interest

The authors have not declared any conflict of interest.

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