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L-asparaginase gene - a therapeutic approach towards drugs for cancer cell

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Abstract

L-asparaginase is an enzyme that reduces the activity of L-asparaginase (an important nutrient for cancer cells) resulting in cancer/tumor cell starvation). L-asparaginase is relatively wide spread enzyme found in many plant tissue, bacteria, plant and in the serum of certain rodents, but not of man. It is used mainly for the induction of remission in acute lymphoblastic leukemia. Although there are therapeutic asparaginases present in the market, recent discoveries have indicated that the L-asparaginase from *Erwinia carotovora* (ErCAR) might be more efficient and also to exhibit fewer side effects. The need for new therapeutic enzymes is of great interest in both biotechnology and medicine. This paper is an attempt to comprise detailed information of the work on the L-asparaginase gene from different sources for the treatment of cancer cells.

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Introduction

Many enzymes have been used as drugs like for the treatment of especially Acute Lymphoblastic Leukemia (ALL) and Lymphosarcoma Cancer Cells (Head, 1995). L-asparaginase enzymes (L-asparaginase amidohydrolase; EC 3.5.1.1) catalyze the hydrolysis of L-asparaginase (L-Asn) to Laspartate (L-Asp) and ammonia (NH_3), and to a lesser extent the hydrolysis of L-glutamine (L-Gln) to L-glutamate (L-Glu) (Fig. 1). Two types of bacterial L-asparaginases have been identified: type I and type II (Campbell *et al*., 1967). Type I L-asparaginases are expressed constitutively in the cytoplasm and catalyze the hydrolysis of both L-Asn and L-Gln, whereas type II L-asparaginase are expressed under anaerobic conditions in the periplasmic space of the bacterial membranes and display higher specificity for L-Asn hydrolysis (Campbell *et al*., 1967; Cedar and Schwartz, 1968).

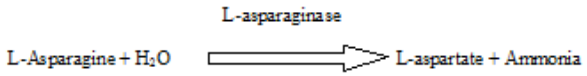

Fig. 1. Hydrolysis of L-asparagine to L-asparatate.

L-asparaginase is very essential amino acid for the growth of tumor cells whereas the growth of normal cell doesn't of its requirement (Berenbaum *et al*; 1970). It can be produced within the cell by an enzyme called Asparagine synthetase. Most of the normal tissue synthesizes L-asparagine in amounts for their metabolic needs but the Cancer or Cells (especially Malignant and Carcinoma Cell) require external source of L-asparaginase for their growth and multiplication(Broome, 1963). In the presence of LA, the tumor cells deprived of an important growth factor and they may failure to survive. Thus this enzyme can be used as a chemotherapeutic agent for the treatment of ALL (mainly in children) as a potent antitumor or antileukematic drug(Nachman *et al.,* 1998). L-asparaginase-II is an important enzyme as therapeutic agents used in the treatment of Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, Lymphosarcoma treatment, reticlesarcoma and melanosarcoma (Steacher *et al*., 1999; Verma *et al*., 2007). L-asparaginase is relatively wide spread enzyme found in many tissue, bacteria, plant and in the serum of certain rodents, not of man. The microbial source are very common for L-asparaginase, because they can be easily cultured and extraction purification of L-asparaginase from them is also convenient, facilitating for the Industrial scale production. The most commonly used microorganism to produce L-asparaginase are *Erwinia caratovora, Bacillus sp. Corynebacterium glutamicum, Pseudomonas stutzeri and E. coli.*(Howard 1968) L-asparaginase from *E .coli* has excellent power to inhibit the activity of tumor cells, and that from *E. chrysanthemi* is also pharmacologically active(James *et al.,* 1970). L-Asparaginase also plays a very crucial role in the biosynthesis of the aspartic family of amino acids. *Corynebacteria* producing amino acid are of great industrial interest as they excrete large amount of various amino acid (Martin, 1989). Methionine, Threonine, and Lysine commercially important amino acid produced by *C. glutamicul,* are derived from aspartic acid (Vernour, 1994). Recent studies have indicated that the expression rate of L-asparaginase gene is very low or slow and their demands are ever increasing hence there is always short supply to Pharmaceutical Company. As production rate is low, the cost of enzyme is very high and is not available to many of the patients.

Mechanism of antineoplastic action of l-asparaginase

The effective depletion of L-asparaginase results in cytotoxicity for leukemia cells (Fig. 2) FDA has approved that such type of drug can be used for the effective treatment of Acute Lymphoblastic Leukemia (ALL) and Lymphosarcoma. Therapeutic enzymes from other drugs are two main features; firstly that the enzymes act on their target with a great specificity and with high affinity, secondly they are catalytic and able to convert a substrate into a desired product(Michel 2003). These features render possible the production

of potent drugs that could carry out therapeutic biochemistry in vivo. Asparaginase are expressed in many bacterial organisms, but only L asparaginase from *Escherichia coli* (*E. coli*) and *Erwinia chrysantemi* (ErCHR) have been used as chemotherapeutics in Acute Lymphoblastic Lymphoma (ALL (Mashburn 1964). Several brand name of L-asparaginase are available in the market such as CLOLAR, ARRANON, LEUKINE, KIDROLASE, ONCASPAR, ELSPAR, and ERWINASE.

Table 1(a). List of L-asparaginase producing microorganism from Bacteria.

Bacteria	References
Acinetobacter calcoaceticus	Joner(1976)
Bacillus sp.	Mohapatra *et al.* (1995)
B.mesentericus	Tiul panova *et al.* (1972)
B.polymyxa	Nefelova *et al.* (1978)
Citrobacter sp.	Bascomb *et al.* (1975)
Corynebacterium glutamicum	Mesas *et al.* (1990)
Escherichia coli	Netrval (1977)
Enterobacter aerogenes	Mukherjee *et al.* (2000)
E.cloaceae	Nawaz *et al.* (1998)
Erwinia aroideae	Tiwari & Dua (1996)
E.carotovora	Maladkar *et al.* (1993)
E.chrysanthemi	Moola *et al.* (1994)
Helicobacter pylori	Stark *et al.* (1997)
Klebsiella pneumonia	Reddy & Reddy (1990)
Mycobacterium phlei	Pasterzak & Szymona (1976)
Pseudomonas ovalis	Badr & Foda (1976)
P.stutzeri	Manna *et al.* (1995)
Serratia marcescens	Rowly & Wriston (1967)
Staphylococcus sp.	Mikucki *et al.* (1977)
S.aureus	Rozalska & Mikucki (1992)
Streptococcus albus	Reddy & Reddy (1990)
Tetrahymena pyriformis	Tsirka (1990)
Thermus thermophilus	Pritsa *et al.* (2001)
T.aquaticus	Curran *et al.* (1986)
Vibrio succinogenes	Disteasio *et al.* (1976)

Microbial sources of L-asparaginase

Over last 35 years various microorganism such as yeast, algae, plants, fungi, actinomycetes are major source of L-asparaginase summarized in Table1 (a) - 1 (b).

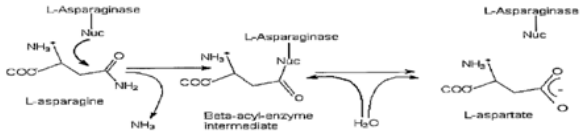

Fig. 2. Schematic illustration of the reaction mechanism if L-asparaginase. The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack (Sanson and Jaskolski, 2004).

L-Asparaginase by bacteria

There are many reports regarding the presence of L-asparaginase in various distinct bacterial source such as *Escherichia coli,*(Netrval 1977) *Erwinia aroideae* (Tiwari and Dua, 1996) and most of the work has been carried out with gram negative bacteria such as *Vibrio succinogenes* (Kafkewitz & Goodman,1974), *Thermus thermophilus* (Prista *et al*., 2001). L-asparginases has also been studied from marine bacteria (Benny & Kurup, 1991) which are considered to be an important source of bioactive enzymes (Williams and Vickers, 1986). Marine bacteria have halophilic in nature, can be used in industrially. L-asparginases production has also been reported in *Pseudomonas flourescens* (Mardashev *et al* 1975.). L- asparaginase production in *Staphylococci* has been described by Mickucki *et al.* (1977).Most of the Industrial researcher as well as Microbiological scientists are preferred to worked with *Tetrahymena pyriformis* (Tsirka 1990), because of its maximum activity of the enzyme has been found in stationary phase of growth and mostly activity has been associated with the ER.(Trianfolliou *et al*., 1988). L-asparginases from a new *Erwinia sp*. has been reported by Bokotky and Bezbaruah (2002).

L-Asparaginase from yeast

L- asparaginases are currently in use are obtained from various member of Yeast specially *Saccharomyces cervisiae* which is encoded by the ASP3 gene (Bon *et al.,* 1977). L-asparginases was also isolated from the cell culture broth of *Candida utilis* (Kil *et al.,* 1995). The production of L- asparaginase has been also reported from *Pichia polymorpha,* was isolated form Egyptian Soils by Enrichment method (Foda *et al.,* 1980). [Table 1(b)]

L-Asparaginase from fungi

Wide range of fungi strains are efficient producers of L- asparaginase. A strain *of A. terrus*, isolated from decomposing of vegetable substrate (Ali *et al.,* 1994) can be used as a better source of L-asparginases production form fungi source. L-asparginases has been studied in *Aspergillus nidulans* (Drainas and Drainas, 1985), *Mucor Sp.* (Mohapatra *et al.,* 1997) and *Cylidrocapron obtusisporum* (Raha *et al.,* 1990).

L-Asparaginase from actinomycetes

Mostafa *et al* reported (1979) has reported that several Actinomycetes were present in different strains (*S. Karnatakensis and S. venezuelae*), were isolated from soil under different environmental and nutritional parameters. Gunasekaran *et al.* (1955) has given the report of L- asparaginase production by *Nocardia Sp. Streptomyces sp* .is theanother source of L-asparaginase, can isolated from the gut of fish *Therampon jarbua* and *Villorita cyprinoids* has L-asparaginase activity (Dhevendaran and Anithakumari, 2002).

Table 1(b). List of L-asparaginase producing microorganism from different Microbial Sources.

Source (Reference)			
Fungi	**Actinomycetes**	**Yeast**	**Algae**
Aspergillus nidulans (Drainas & Drainas, 1985)	*Sterptomyces karnatakensis* (Mostafa, 1979 a)	*Candida utilis* (Kil *et al.* 1995)	*Chlamydomonas sp.* (Paul, 1982)
A.terreus (Ali *et al.,* 1994)	S.venezuelae (Mostafa , 1979 b)	*C.guilliermondii* (Stepanyan and Davtyan, 1988)	
Cylidocapron obtusiporum (Raha *et al.,* 1990)	*S.collinus* (Mostafa and Salama, 1979)	*Pichia polymorpha* (Foda *et al.,* 1980)	
Mucor sp. (Mohapatra *et al.* 1997)	*Thermusactinomyces vulgaris* (Mostafa & Ali, 1983)	*Saccharomyces cerevisiae* (Bon *et al.,* 1997)	

L-Asparaginase by plant source

Like microbial source, plants are also source of L-asparaginase. *Lupin arabreus* such as leaves, flower buds, root tips and *Lupin angustiplius* which has the ability to produced L- asparaginase (Borek *et al.,* 1999). L- asparaginase, activity has also been reported in soil of roots of *Pinus pinaster* and *Pinus radiate* (Bell and Adams, 2004).

Properties of L-asparaginase

Several parameters such as Temperature, pH, Oxygen, several chelating agents such as EDTA play a very key role for the maximum growth of the enzyme producing organism (Mesas *et al.,* 1990). A trace element like Metal ions doesn't affect the production of L-asparginases. Some of the agents like 2-mercaptoethanol, glutathione enhance the activity of enzyme (Raha *et al.,* 1990). Physic-chemical may vary due to their different source of L-asparginases.

Generally L-asparginases from Guinea pig serum have pH 7.5-8.5 and molecular weight of 1,38,000 Da. It is stable for at least 6 months at 20° C to repeated freezing and thawing and to heating to 55 °C for 10 min, which promotes the surface denaturation (Mishra, 2006).

Production of microbial L-asparaginase

Several research reports about the production of L-asparginases, can be produced from different source of microorganisms either produce this enzyme constituently or after induction. Several parameters especially physical and chemical parameter for L-asparginases production vary with the species of microbial source (Barnes *et al.,* 1977). Other constituents such as media composition can affect the growth as well as production of L-asparaginase of *V. succinogenes* was studuies (Albanase and Kafkewitz, 1978). *S.cerevisiae* synthesizes two form of asparaginase, L-asparaginase-I is constitutive and L-asparaginase-II is secreted in response to N starvation (Dunlop *et al.,* 1978). L-asparginases showed that carbon Source such as sucrose, glucose, maltose, galactose,mannitol and mantose inhibited while exogenous c-AMP in the presence of Carbon source stimulated, L-asparaginase enzyme (Rozalska and Mickucki 1992). ,High activity of L-asparaginases has been reported in bacterial culture, were growing in ample nitrogen (Paul and Cooksey, 1981). In the presence of glucose, activity of L-asparaginase from *E.coli-W* and *E.coli K-12* has been completely suppressed. This was because glucose caused catabolite repression and catabolite inhibition of the components involved in the lactate transport (Garaev and Golub, 1977) and lactate stimulated L-asparaginase synthesis. Some of the Organic acid and Amino acid especially such as L-methionine and L-leucine were found to enhance production of L-asparaginase in *E. coli* (*Netrval,* 1977). 12 Carbon and 21 Nitrogen source was used for production of L-asparginases by *Enterobacter aerogene,* C and N sources can be used in the form sodium citrate and di-ammonium hydrogen phosphate *(*Mukherjee *et al.,* 2000*)*.

L-asparaginase is produced by submerged fermentation (SmF). This methodology has some limitation like net yield is low and cost intensive. Another alternative solution to SmF is solid state fermentation (SSF) which is offering a wide range of advantages compared to SmF(Lonsane *et al.,* 1985). SSF methodology is a very effective technique, as the yield of the product is many times higher when compared to that in SmF6, and it also offers many other advantages. Glucose was a repressor and Nitrogen catabolite repression on enzyme formation was absent in this bacteria of this biosynthesis. Dissolve Oxygen (KL_a) play a key role during production of LA through Solid State fermenter, Dissolve oxygen was very limited. *Staphylococci* had the maximum yield during the stationary phase of growth on a batch culture where Carbon and Nitrogen Sources can be supplied as Casein hydrolysate and yeast extract (Mikucki *et al* 1997). Maximum yield of LA was found when culture were aerated during the accelerated log phase or exponential phase of growth and further incubated in the stationary phase. Repression by L-asparaginase and L-aspartic acid was absent but glucose inhibited the enzyme formation (Savitri *et al* 2003).

Various types of media can be used as N and C source especially synthetic media with have maximum production than natural media by *Streptomyces (*Mostafa and Salama, 1979*)*. For the production of maximum yield of L-asparginases, Starch (1.0%) as Carbon and asparagines (0.8%) as Nitrogen source was optimum for enzyme production at pH 8.5. Incubation was done at 28-30 °C for six days.

S. cerevisiae has ability to produced L-asparaginase under Nitrogen Starving condition (Bon *et al.,* 1997). Production of L-asparginases is depending upon the functional gene GLN3 and that the response to N

availability is under control of gene product URE2 (Eliba *et al.,* 1997). L-asparginases produced by cultivating the cells of *Candida utilis* medium containing glucose, yeast nitrogen base and peptone at 30°C. After 18 hours, Cells were collected by centrifugation and L-asparginases activity was measured (Kil *et al.,* 1995).

Recombinant L-asparaginase

Several methods are available to producing L-asparaginase commercially by using the modern biotechnological approaches such as R-DNA technology, gene cloning etc. Presently, L-asparaginase is produced throughout the world by submerged fermentation (SmF). This methodology has many disadvantages such as the low concentration product formation and consequent handling, reduction and disposal of large volumes of water during the downstream processing etc. Therefore, the SmF methodology is a cost intensive, highly problematic and poorly understood unit operation. An alternative solution to Molecular Cloning and Genetic Engineering are the promising key tools which has ability to produced Recombinant L-asparaginase. Henry *et al* (1986) cloned and produce Recombinant L-asparaginase through expressed *E. crysanthemi* asparaginase gene in *E.coli* and *Erwinia carotovora*. The enzyme was produced at high level in *E.coli* (0.5% of soluble protein) and was shown to be exported to periplasmic space. Expression of cloned gene was subjected to glucose repression in *E.coli.* but was not significantly repressed by glycerol. The isolated *Erwinia* asparaginase gene was successfully introduced in *E. carotovora* and enzyme expression was approximately three-fold higher than the production strain of *E.crysanthemi* (Aghaiypour *et al.,* 2001).

Spring *et al.* (1986) were studied *E. coli* mutant resistant to substrate of L-asparaginase. It was found that the gene encoding L-asparaginase I and L-asparaginase-II both were having different sequence and are not sequence related. Cloning of *E.coli* gene ans B encoding L-asparaginase –II was completely based on the PCR amplification and sequencing was discussed by Bonthron (1990). In plants, especially *Lupin arborens,* isolation and characterization of cDNA encoding L-asparaginase from the developing seed have been reported by Lough *et al.* (1992). Dickson *et al.* (1992) has also reported the molecular cloning of the gene encoding developing seed L-asparaginase from *Lupin angustifolius.* Expression of L-asparaginase –II encoded by ans B in *Salamonella enteric* was found to pe positively regulated by a cAMO receptor protein (cRp) and anaerobiosis (*Jennings and Beecham,* 1993). Recombinant L-asparaginase was also studied epotopes on *Erwinia chrysanthemi* using synthetic hexapeptides and polyclonal antiserum from rabbits and mice (Moola *et al.,* 1994). Elimination of immunodominant epitope in the enzyme by Site directed mutagenesis resulted in markedly decreased binding of the antibodies indicating reduced immunogenicity while the enzyme activity remained unchanged.

Cloning and expression studies of L-asparaginase in *E.coli* has been reported, Cloning was done as a DNA fragment generated by PCR. The recombinant plasmid PASN, containing asparaginase gene using expression vector PBV 220, was transformed in *E.coli* host strains. Higher activity was found in recombinant enzymes. Recombinant L-asparaginase from *Erwinia carotovora* expressed in *E. coli* and purified was reported by Borisova *et al.* (2003). Large quantity of L-asparaginase mRNA was measured by RQ-PR as described by Irino *et al.* (2004). The AS mRNA level paralleled the AS enzyme activity and the as protein level. Krasotkina *et al.* (2004) was used chromatography technique for purification of Recombinant L-asparaginase from *Erwinia carotovora.* The kinetic properties show that recombinant L-asparaginase combined the main advantages of *Erwinia chrysanthemi* and *E. Coli* L-asparaginase –II. Kotozia and Labrou (2005) reported

that recombinant L-asparaginase was produced by cloning L-asparaginase form *Erwinia carotovora* NCYC 1526 (Er A) and expression was done in *E. coli,* purification was carried out by anion exchange chromatography and affinity chromatography. The kinetic parameters (K_m and V_{max}) of the enzyme were also estimated. Recombinant humans Asparginase , *i.e.* C-terminally tagged, has been prepared in a *Baculovirus* based expression system. The recombinant enzyme has higher catalytic activity and offers a major possibility in identifying and characterizing inhibitors that may be used as asparagine resistant cells (Ciustea *et al.,* 2005). Expression of recombinant L-asparaginase fused to pub leader sequence under the inducible T_7 lac promoter in BLR (DE) host cells resulted in optimum extracellular production in shake flasks. The enzyme had 80% activity of the native enzyme (Khushoo, Pal and Mukherjee, 2005). Recombinant L-asparaginase from *Erwinia chrysanthemi* 3937 (Erl-ASNase) has been expressed in *E.coli* BL21 (DE3)pLysS (Kotzia and Labrou, 2006). Several result were examined like enzymatic, structural properties, and kinetic parameters [K(m), k(cat)] for a number of substrate. The enzyme was later immobilized on epoxy-activated Sepharose CL-6B. The immobilized enzyme retained most of its activity (60%) and showed high stability at 4° C.

Conclusion

Production of L-asparaginase using different microbial systems has attracted much attention, owing to the cost-effective and eco-friendly nature. A wide range of microorganisms including fungi, yeasts and bacteria have proved to be the beneficial sources of this enzyme. L-Asparaginase is an important natural product that possesses a broad spectrum of antitumor activity. It has been successfully applied to the treatment of several diseases such as lymphocyte sarcoma and leukemia. Recent studies have indicated that the expression rate of L-asparaginase gene is very low or slow and their demands are ever increasing hence there is always short supply to pharmaceutical company. As production rate is low, the cost of enzyme is very high and is not available to many of the patients. By using latest methods like Genetic Engineering, Molecular cloning have a powerful novel tool which increases the net yield of production rate for L-asparaginase. Biotechnological advancements have enabled for enhanced potency and specificity among enzymes with a production at a lower cost. Therapeutic enzymes have a broad variety of specific uses as oncolytic, anticoagulants or thrombolytic, and as replacements for metabolic deficiencies. The need for new therapeutic enzymes is of great interest in both biotechnology and medicine.

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