Review Article

Important Sources and Medicinal Applications of L-Asparaginase

*Hassan Mustafa Arif, Zahid Hussain

Institute of Industrial Biotechnology, GC University, Lahore, Pakistan.

ABSTRACT

L-Asparaginase (EC 3.5.1.1) is an amidohydrolase which catalyzes the breakdown of L-asparagine to ammonia and L-aspartic acid. Its broad use in both: food industry and chemotherapy makes it an object of wide research. It can reduce acrylamide formation in the baking of starchy foods and selectively kill tumor cells by depleting asparagine levels in the blood which is essentially required by tumor cells for continuous growth. However, hypersensitivity and toxicity puts a big question mark on L-Asparaginase therapy and raises the need to research for the ideal enzyme which should exhibit lower antigenicity, toxicity and a higher efficacy. Thusall future research should be directed towards lowering or eradicating of the undesirable properties to produce such enzyme for therapy. Some of the promising methods include: Site directed mutagenesis to remove immunogenic epitopes from the enzyme, using Random mutagenesis to increase efficacy of the enzyme or using Poly-L-Asparaginasenano capsules for increased efficacy and delivery with reduced toxicity. Although this subject has been reviewed in the past but an updated review with a comparison with previous studies can serve as fuel and direction for future research. The following review discusses the current research on L-asparaginase, its properties, sources, uses and future implications.

Keywords: Acrylamide, acute lymphoblastic leukemia, GRASPA, L-Asparaginase, PEGasparagase.

Received 25 April 2014 Received in revised form 13 June 2014 Accepted 15 June 2014

***Address for correspondence:**
Hassan Mustafa Arif,
Institute of Industrial Biotechnology, GC University, Lahore, Pakistan.
E-mail:microbiologisthassan@gmail.com

INTRODUCTION

L-Asparaginase (L-asparagine amido-hydrolase; EC 3.5.1.1), belongs to the amidohydrolase family. It catalyzes the breakdown of L-asparagine to ammonia and L-aspartic acid. The ammonia produced can be quantified using a gas analyzer with ammonia sensing electrodes (1) or the ammonia can be combined with α-ketogluterate to form L-glutamic acid using glutamate dehydrogenase. This reaction is marked with a decline in the NADH concentrationand is detected as a loss of absorbance at 340 nm. Thus the activity of L-Asparaginase can be determined and expressed as ASNU unites where one ASNU is defined as "The amount of L-Asparaginase that produces 1 micromole ammonia per minute under the conditions of the assay (pH=7±0.05;temperature= 37.0 ±0.5 ºC) (2).

L-Asparaginase has two broad applications, one of which is in the food industry (3) where L-Asparaginase is usedin the production of dough based products, such as cookies, and high starch containing products such as potato chips and french fries(4). This is done to reduce acrylamide formation which takes place at elevated temperatures of baking or frying as a consequence of millard reaction between Asparagine and the reducing sugars present in such food (3, 5, 6). The other application of L-Asparaginase is as an antineoplastic agent used in the treatment of acute lymphoblastic leukemia and lympho-sarcoma (7). L-Asparagine is required by a normal growing cell for protein synthesis. It is either acquired externally through diet or synthesized by the cell using L-Asparagine synthetase. Tumor cells have decreased L-Asparagine synthetase expression and their dependence on high amounts of external L-Asparaginefor protein synthesis to keep up with the rapid proliferation rates is

exploited in L-Asparaginase therapy. The enzyme deprives tumor cells by depleting external L-Asparagine, resulting in their death (8, 9).

Isolation of L-Asparaginase

L-Asparaginase can be isolated from plant and microbial sources.

From *E. coli*

E. coli produces two distinct L-Asparaginases differing in properties, especially in affinities for the substrate asparagine (10). L-Asparaginase II has a higher affinity for asparagine and is localized in the periplasmic space between the cell membrane and cell envelope (11). L-Asparaginase II has been shown to inhibit the growth of rat and mouse tumors grown in vivo more effectively than L-Asparaginase I which is ineffective against such tumor growth(12-14).

It was concluded by (10) that the optimum production of L-Asparaginase II takes place when bacteria is grown at pH 7 to 8 at 37 ºC in an enriched media with variety of amino acids in high concentration with no specific amino acid inducing L-Asparaginase synthesis. It was also shown that glucose along with other sugars has an inhibiting effect on enzyme synthesis. The enzyme production was seen between the transitions from aerobic to anaerobic growth. Although enzyme was produced in aerobic conditions; however, 100-1000 times more L-Asparaginase was produced anaerobically.

From *Erwinia*

L-Asparaginase purified from *Erwinia aroideae* overcomes the problem of antigenic reactions taking place in some patients under treatment with L-Asparaginase II of *E. coli* (15-17). Although L-Asparaginaseisolated from *E. coli* and *Erwinia* are similar in size (approximately 140,000), they are not homologous (18-20).

Peterson and coworkers in 1969 concluded that*Erwinia* has only one, intracellular L-Asparaginase and by using *Erwinia aroideae* NRRL B-138, maximum obtainable yield of L-Asparaginase was 1250 IU per gram of dry weight of cells in 8 hours. After extraction, partial purification and concentration a yield of 275 IU/ml was obtained. The pH optimum for enzyme was found to be 7.5 with a Km of 3 X 10^{-8} M. L-Asparaginase from *Erwinia*possessed strong antitumor activity as a dose of 3.6 IU caused tumor regression up to 6 mm in 4 days and only a single dose of 5IU injected intraperitoneally into mice yielded complete remission (16).

By other bacteria

Peterson and coworkers in 1969 furthertested 123 bacterial isolates for L-Asparaginase production. They found enzyme yields between 80-950 IU per gram dry weight of cells. They further concluded that almost all strains tested had some measureable amount of enzyme; however, one strain of *Hydrogenomonas eutropha* and four strains of *Erwinia aroideae* produced substantially higher amounts of the enzyme under similar conditions. Other bacteria tested included *Pseudomonas* with highest yield shown by *P. aureofaciens* (up to 300 IU/g); *Proteus* with highest yield by *P. vulgaris* (up to 370 IU/g); *Serratia marcescens* (up to 335 IU/g)(21).

From Filamentous fungi

L-Asparaginase from bacterial sources has the problem of causing allergic reactions (22). Pronk and coworkers in 2008concludedL-Asparaginase of eukaryotic origin (*Aspergillus oryzae*) has lower allergenicity (2). Production of L-Asparaginase from filamentous fungi was reported bySarquis and coworkers in 2004 (23). They investigated L-Asparaginase production in *Apergillus tamarii* and *A. terrus*. It was seen that *A. terrus* exhibited highest activity (58 U/L) in 2% proline medium and no activity in medium containing glutamine and urea as nitrogen sources, suggesting that L-Asparaginase production is under nitrogen regulation.

Other fungi known to show L-Asparaginase activity include: *Penicillium, Fausarium, Rhodotorula* and*Rhodosporidium* (24-29).

From plants

Heeshenand coworkers in 1996 discovered two isoforms of L-Asparaginase from Bryophyte *Sphagnum fallax* (30). Similarly, Borek and cowerkers, in 1999noted*Lupin araboreus* and *Lupin angustiplius* showed greater production of L-Asparaginase (31). The enzyme is also found in roots of *Pinus radiata* and *Pinus pinaster* due to ectomycorhizal association (32). Bruneauand coworkers in 2006found a K^+

dependent L-Asparaginase in *Arabidopsis* (33).

Detection, Purification and Characterization

First reports on detection using a simple plate assay for L-Asparaginase activity was made by Gulati and coworkers in 1997 (34), done on modified M9 media and Czapekdox's media supplemented with phenol red as an indicator for pH change which occurs due to the formation of ammonia on the breakdown of L-Asparagine. Pink to red colonies, exhibiting Asparaginase activity can be clearly differentiated from others while the intensity of colour co-relates with the activity. A modified version of the assay was reported byGhasemi and coworkers in 2008 (35).

Purification and Characterization of L-Asparaginase from *Vibrio succinogenes* was documented first byDistasio and cowrkers in 1976 (36). They first obtained a homogeneous preparation of L-Asparaginase by ammonium sulfate fractionation and then isolated the enzyme by affinity chromatography on columns of hydoxylapatite, CM-Sephadex and DEAE-Sephadex, respectively. The purity of L-Asparaginase was tested on SDS - gel electrophoresis which yielded a single band. The overall yield obtained by them was 40 to 45 % with a specific activity up to 200 IU per mg of protein. The enzyme had a molecular weight of 146,000 with a Km of 4.78 X 10^{-5} and did not carry any disulfide, sulfhydryl, tryptophan, and phosphorus or carbohydrate groups. L-Asparaginase of *V. succinogenes* hydrolyzed D-Asparagine at a rate of 6.5% greater than with the L isomer and is immunologically distinct from *E. coli*L-Asparaginase.

Most common way of purification is through ammonium sulfate precipitation followed by chromatographic techniques.Nagarethinam and coworkers in 2012 compiled various techniques used with results expressed as purification fold in their review (8).

Crystal structure of L-Asparaginase

Crystallographic investigation of L-Asparaginase II is recorded bySwain and coworkers in 1993 (37). They determined Elspar at 2.3 Aº resolution by combining molecular replacement with data obtained a single heavy atom derivative. The enzyme was found to be a homo-tetramer belonging to α/β class of proteins with 222-symmetry. There are two subunits on each domain with unique topological features. They proposed the active site between C and N-terminal domains belong to different subunits and suggested a role of Thr-89 in catalysis. In this regard, the role of Thr-15 and Try-29 in catalysis was confirmed byAghaiypour and coworkers in 2001 (38). A comparison between solution and crystal form of *E. coli*L-Asparaginase II by SAXS pattern was performed byKozak and coworkers in 2002 (39). They found that the overall quaternary structure is same in both crystal and solution form; however, the homo-tetramer is less compact in solution than in the crystal form.

L-Asparaginase from *Erwinia chrysanthemi* (ErA) was determined at 1.8 Aº resolution by Miller and coworkers in 1993 (40). They found a rare occurring in its structure; pair of dimers were linked by conserved residues in a left-hand crossover into a tetrameric, catalytically active form, which was essentially responsible for release of product. Palm and coworkers in 1996 reported that the nucleophile, Thr15 is located in the flexible region (41).

K^+independent plant L-Asparaginases were reported byMichalska and coworkers in 2006 (42). They found that the αβ subunits arise from auto-proteolytic cleavage of two copies of precursor protein and the heterodimer is in αββα configuration. Thr-193 acts as the nucleophile and is part of the active site. The unusual loop seen in bacterial L-Asparaginases is also present in plant enzyme.

As a food agent

As documented in a joint FAO/WHO report in 2007, asparaginase enzyme is used in food industries to prevent acrylamide formation in dough-based products, and starchy food, such as cookies and french fries. Acrylamide is formed by Maillard reaction between asparagine and reducing sugars which are abundantly present in such foods. This reaction is prevented by adding asparaginase which converts asparagine into aspartic acid, making it unavailable for Maillard reaction. For

dough-based products the enzyme is added prior to baking in 200-2500 ASNU (0.06-0.7 g enzyme preparation) per kilogram food and potato strips are dipped in 2000 ASNU (0.6g enzyme preparation) per killogram food (3).

Acrylamide, a notable carcinogen, has high concentration in heat-treated foods abundant in carbohydrates (5). In this context,Boegl in 2006, tested the potency of Asparaginase to reduce acrylamide formation in potato products (4). They found that even small amounts, such as 0.2 units per gram of fresh potato was sufficient enough to decrease acrylamide production by 50% and higher doses up to 1 unit per gram lead to increase in acrylamide reduction, almost up to 97%. Similarly,Anese and coworkers in 2011tested the effect of asparagine in reducing acrylamide formation in biscuits (6). From their experiments they concluded that at intermediate concentrations of asparaginase acrylamide formation was considerably reduced with no significant change in colour or taste of the final product.

As a drug

Tumor cells require abundant amount of L-Asparagine to grow. They rely on asparagine from diet as well as asparagine synthesized by the cell itself. L-Asparaginase converts L-Asparagine to aspartic acid and ammonia, decreasing the L-Asparagine for tumor cells which heavily rely on L-Asparagine from serum as they have lower levels of L-Asparagine synthetase expression (8, 43, 44). Asselin and coworkers in 1989 quantified the ability of L-Asparaginase as a single agent, in vivo and in vitro, in killing tumor cells in patients undergoing treatment for acute lymphoblastic leukemia (45); cell cycle arrest was observed in murine L5179Y cell line (46) and MOLT-4 human T-lymphoblastoid line (47), causing cell death. *Erwinia caratovora*L-Asparaginase significantly inhibited a human acute lymphoblastic leukemia cell line (48).

The anti-tumor activity of L-Asparaginase is related to the circulation half-life of the enzyme in host serum (49) and its affinity for L-asparagine (12). Among bacterial sources, *E. coli*L-Asparaginase and *Erwinia carotovora* L-Asparaginase have sufficient circulation half-lives to be of clinical importance (19, 50, 51). In the pharmacokinetics of the drug, it has been observed that after administration the drug remains localized to vascular spaces. It is detected in ascities and pleural fluids (52) but not in cerebrospinal fluid (53). Schwartz in 1970found out that after administration of 5000IU/Kg body weight dose, only a small level of enzyme was detectable in cerebrospinal fluid and after injecting directly in cerebrospinal fluid, the enzyme was immediately transferred to plasma (54). Many investigators have proved that patients with hypersensitivity reactions to native L-asparagine preparation tend to have a decreased half-life for L-Asparaginase(12, 55-58).

Clinical pharmacology of L-Asparaginase was reported by Ho and coworkers in 1981 (59). They in injected 10,000 U/m^2intramuscularly and 16,500-100,000 U/m^2 intravenously to patients with metastatic cancer and found that the plateau in plasma after intramuscular injection was reached by 14-24 hours while peak plasma levels remained 1.12 U/ml which is one-fourth the activity reached when compared with same dose given intravenously.

Resistance to L-Asparaginase

L-Asparaginase was first believed to be effective against cancerous cells without affecting normal cells. However, this belief was falsified when resistance to the drug emerged by de-repression of L-Asparaginase synthetase gene (60, 61). An increase in L-Asparaginase synthetase activity was observed in patients with acute lymphoblastic leukemia and murine lymphoma cells (44,56,62).

Another reason for resistance includes immergence of specific antibodies which rapidly clear L-Asparaginase levels (63). Results of experimentsdemonstrated normal levels of L-Asparaginase in plasma after drug administrations in patients with hypersensitivity to *E. coli* and *Erwinia* L-Asparaginase(45, 64, 65).Killander and coworkers in 1976found drug efficacy remained unimpaired even after immunization of patients (66).

Gallagher in 1989believed that sensitive cells control the expansion of resistant cells via cytokines (67).

Toxicity

Toxicity from L-Asparaginase ranges from acute hypersensitivity and hyperglycemia to pancreatitis and hepatocellular dysfunction (68). The toxicity can be either due to adverse effects of inhibiting protein synthesis or due to hypersensitivity. PEGylation has been reported to reduce toxicity. Generally, L-Asparaginase causes little bone marrow depression and usual dosage does not affect the gastrointestinal or hair follicles or oral mucosa (67).

Clinical manifestation of hypersensitivity involves urticaria; however, allergic reactions range from localized erythema to systemic anaphylaxis. In some patients induration, swelling, edema, chill, tenderness, fever and even skin rashes are reported (69) and often require discontinuation of the drug. Jaffe and coworkers in 1973calculated the risk factor for L-Asparaginase hypersensitivity and found it to be above 6000IU/m^2 per day with intravenous route of administration (70). Although desensi-tization methods were described but the sample size was not statistically significant (71).

Rivera-Rodriguez and coworkers in 2013 tested delivery of the enzyme through Poly-L-Asparaginasenanocapsules and found this method to be significantly less toxic (72).

Modified L-Asparaginase

In approximately 25% of the patients, allergic reactions ranging from mild to anaphylactic shocks were observed (73). This created the need for development of L-Asparaginase with decreased potential immunogenicity but at the same time preserving its activity. Thus, in mid-1970s, the search for such methods begun (74, 75). Most promising method found was the conjugation of PEG with L-Asparaginase (PEGylation). Two independent teams in 1979 led by Abuchowski and Uren were the first ones to have success in this regard (74, 75). They successfully conjugated PEG with L-Asparaginase and found that the conjugated enzyme had a significantly decreased immunogenic response. Pegylated L-Asparaginase has a higher molecular weight (76) and has antitumor activity in both animals (74, 77) and humans (77, 78). However studies conducted by Armstrong and coworkers in 2007 showed the presence of Antibodies against PEG causing rapid removal of Pegylated L-Asparaginase, thus warranting the need for screening of anti-PEG antibodies during therapy (79).

Many other procedures were also tried and tested such as coupling with dextran which increased stability but had no effect on immunogenicity (75, 80); *Erwinia* L-Asparaginase with Human serum albumin lack proper study (81); Acylation had the limitation that enzyme becomes hydrophobic after modification (82),Gilbert and coworkers in 1986successfully cloned and expressed *Erwinia crysantemi*L-Asparaginase in *E. coli* and *Erwinia carotovora* (83). This proved to be a successful technique in reducing immunogenicity. Moola and coworkers in 1994 removed immunogenic epitopes by site directed mutagenesis (84). This also proved to be a successful technique in significantly reducing immunogenicity.

Other methods, such as entrapment in RBC increased stability but had no effect on immunogenicity (82). Kwon and coworkers in 2009 used "membrane-translocating low molecular weight protamine (LMWP)" to introduce L-Asparaginase into the red blood cell without disrupting its membranes reported significantly improved the half-life of the enzyme to 4.5 ±0.5 days (85).

Clinical trials

Clinical trials of L-Asparaginases of *Erwinia* and *E. coli* have been discussed over 30 years. Due to hypersensitivity associated with native preparations, modified drugs have more importance in clinical applications. Allergic responses were decreased with PEGylated enzyme which also showed greater half-life in plasma (52, 86,87).

Two independent teams lead by Ho in 1986 and Keating in 1993 first conducted tests; administering 500-800 IU/m^2pegaspara-gase by IV route over 1 hour fortnightly. They observed only three patients developed symptoms of toxicity in the form of anaphylaxis. Other associated toxicities included hyperglycemia and hepatic dysfunction. Subsequent trials were

undertaken with 2000 and 2500 IU/m^2 for clinical studies (88, 89).

Vieira Pinheiro and coworkers in 2001, in their trials, infused 500U/m^2 of PEGylated enzyme in children with relapse of acute lymphoblastic leukemia and observed that majority of patients responded well for the first week (90). Another group tested pegaspargase in patients with advanced stage solid tumors by subjecting them with increasing dose of 250, 500, 1000, 1500 and 2000 U/m2 biweekly. Majority of patients showed lower levels of L-asparaginse for 14 days with grade 1-2 hypersensitivity reactions often seen at 2000 u/m2 dose (91).

In a multi-center phaseII trial, 21 patients with acute lymphoblastic leukemia were given a dose of 2000 IU/m2, singly during 14 day investigation period. Thereafter multi-agent therapy was initiated consisting of vincristine, prednisone doxorubicine along with pegaspargase. After the initial period 22% of the patients had complete or partial remission and by the end of 35 days 78% of patients achieved complete or partial remission. Mild urticaria and mild local allergic reactions were seen as only hypersensitivity in five patients (92).

A dose of 2000IU/m^2 were given thrice biweekly in another phase II study of pegaspargase which yielded partial or complete remission in 85.7% of the patients (93).

Randomized tests designed to compare native preparations of *E. coli* with pegaspargase yielded a combined complete and partial response of 65% and 63% respectively. In another randomized comparison between PEGylated enzyme and native enzyme preparation comparable results were obtained with 98% for pegaspargase and 100% for native drug (94).

In current L-Asparaginase therapy, Domenech and coworkers, in 2011 documented randomized trials of GRASPA™ which is RBC entrapped L-Asparaginase made by ErytechPharma Lyon, France. Similarly, a combination of "Dexamethasone, Methotrexate, ifosfamaide, L-Asparaginase, and etoposide (SMILE)" for combating Natural-killer lymphomas (95) entered phase I reported by Yamaguchi and coworkers in 2008 (96) yielding promising results and entered phase II reported by Yamaguchi in 2011 (97).

CONCLUSION

L-Asparaginase has applications in both, medical and non-medical fields. Its rapid use in therapeutics and food industry will raise its demand in the coming years. However, there are still some unaddressed concerns which may require significant research. Immunogenicity due to complex structure is one of the main problems. Although PEGylation may decrease immunogenicity, it does not eliminate it and the anti-PEG antibodies exhibited by some patients further clear the enzyme which renders the therapy ineffective. Other approaches such as, acylation or elimination of different epitopes by site directed mutagenesis should be further researched along with safe delivery mechanisms, such as entrapment in RBC or innanocapsule which would increase the stability and half-life of the enzyme. L-Asparaginase is also reported to have glutaminase activity (98) which may be beneficial due to the fact it enhances L-Asparagine depletion but also causes reduced protein synthesis which is deleterious to the cell and body. Therefore L-Asparaginase without glutmainase activity also needs to be researched for a safer chemotherapy.

Although different microbes have been reported to produce L-Asparaginase at overwhelming levels, however their application in therapeutics or food industry may be limited. The existence of extracellular L-Asparaginase of fungi seems a promising candidate for upscale production of the enzyme as extracellular enzymes are easier to purify and eukaryotic enzymes may prove to be less immunogenic. Strains for ideal production of L-Asparaginase should be researched. An ideal strain would be ubiquitous in nature and produce high yields of L-Asparaginase with simplistic growth requirements. An ideal enzyme would have lower antigenicity and toxicity with a higher efficacy.

REFERENCES

1. Fraticelli Y, Meyerhoff M. On-line gas dialyzer for automated enzymatic analysis

with potentiometric ammonia detection. Analytical chemistry. 1983;55(2):359-64.
2. Pronk MM, Verger P, Olempska-Beer Z, Walker R. ASPARAGINASE FROM ASPERGILLUS ORYZAE EXPRESSED IN ASPERGILLUS ORYZAE. Safety evaluation of certain food additives and contaminants. 2008:55.
3. WHO. Safety Evaluation of Certain Food Additives and Contaminants: World Health Organization, International Programme on Chemical Safety; 2008.
4. BOEGL ZC-EK-P. Impact of L-asparaginase on acrylamide content in potato products. Journal of Food and Nutrition Research. 2006;45(4):141-6.
5. Tareke E, Rydberg P, Karlsson P, Eriksson S, Törnqvist M. Analysis of acrylamide, a carcinogen formed in heated foodstuffs. Journal of agricultural and food chemistry. 2002;50(17):4998-5006.
6. Anese M, Quarta B, Frias J. Modelling the effect of asparaginase in reducing acrylamide formation in biscuits. Food chemistry. 2011;126(2):435-40.
7. Leventhal BG, Henderson ES. Therapy of acute leukemia with drug combinations which include asparaginase. Cancer. 1971;28(4):825-9.
8. Nagarethinam S, Nagappa AN, Udupa N, Rao V. Microbial L-Asparaginase and its future prospects. Asian Journal of Medical Research. 2012;1(4):159-68.
9. Verma N, Kumar K, Kaur G, Anand S. L-asparaginase: a promising chemotherapeutic agent. Critical reviews in biotechnology. 2007;27(1):45-62.
10. Cedar H, Schwartz JH. Production of L-asparaginase II by Escherichia coli. Journal of bacteriology. 1968;96(6):2043-8.
11. Cedar H, Schwartz JH. Localization of the two L-asparaginases in anaerobically grown Escherichia coli. Journal of Biological Chemistry. 1967;242(16):3753-5.
12. Schwartz JH, Reeves JY, Broome JD. Two L-asparaginases from E. coli and their action against tumors. Proceedings of the National Academy of Sciences of the United States of America. 1966;56(5):1516.
13. Campbell H, Mashburn L, Boyse E, Old L. Two L-Asparaginases from Escherichia coli B. Their Separation, Purification, and Antitumor Activity*. Biochemistry. 1967;6(3):721-30.
14. Mashburn LT, Wriston Jr JC. Tumor inhibitory effect of l-asparaginase from< i> Escherichia coli</i>. Archives of Biochemistry and Biophysics. 1964;105(2):450-3.
15. Oettgen H, Old L, Boyse E, Campbell H, Philips F, Clarkson B, et al. Inhibition of leukemias in man by L-asparaginase. Cancer research. 1967;27(12):2619.
16. Peterson R, Ciegler A. L-asparaginase production by Erwinia aroideae. Applied microbiology. 1969;18(1):64-7.
17. Vrooman LM, Supko JG, Neuberg DS, Asselin BL, Athale UH, Clavell L, et al. Erwinia asparaginase after allergy to E. coli asparaginase in children with acute lymphoblastic leukemia. Pediatric blood & cancer. 2010;54(2):199-205.
18. Wade H, Elsworth R, Herbert D, Keppie J, Sargeant K. A new L-asparaginase with antitumour activity. The Lancet. 1968;292(7571):776-7.
19. Cooney D, Handschumacher R. L-asparaginase and L-asparagine metabolism. Annual review of pharmacology. 1970;10(1):421-40.
20. North A, Wade H, Cammack K. Physicochemical studies of L-asparaginase from Erwinia carotovora. 1969.
21. Peterson R, Ciegler A. L-asparaginase production by various bacteria. Applied microbiology. 1969;17(6):929.
22. Reynolds D, Taylor J. The Fungal Holomorph: A Consideration of Mitotic Meiotic and Pleomorphic Speciation. CAB International, Wallingford, UK. 1993.
23. Sarquis MIdM, Oliveira EMM, Santos AS, Costa GLd. Production of L-asparaginase by filamentous fungi. Memorias do Instituto Oswaldo Cruz. 2004;99(5):489-92.
24. De-Angeli LC, Pocchiari F, Russi S, Tonolo A, Zurita VE, Ciaranfi E, et al. Effect of L-asparaginase from Aspergillus terreus on ascites sarcoma in the rat. 1970.
25. ARIMA K, SAKAMOTO T, ARAKI C, TAMURA G. Production of extracellular L-asparaginases by microorganisms. Agricultural and Biological Chemistry. 1972;36(3):356-61.
26. Foda M, Zedan H, Hashem S. Characterization of a novel L-asparaginase produced by Rhodotorula rubra. Revista latinoamericana de microbiologia. 1979;22(2):87-95.
27. Ramakrishnan M, Joseph R. Characterization of an extracellular asparaginase of Rhodosporidium toruloides CBS14 exhibiting unique physicochemical properties. Canadian journal of microbiology. 1996;42(4):316-25.
28. Patro KKR, Satpathy S, Gupta N. Evaluation of some fungi for L-asparaginase production. Indian Journal of Fundamental

and Applied Life Sciences. 2011;1(4):219-21.
29. Nakahama K, Imada A, Igarasi S, Tubaki K. Formation of L-asparaginase by Fusarium species. Journal of general microbiology. 1973;75(2):269-73.
30. Heeschen V, Matlok J, Schrader S, Rudolph H. Asparagine catabolism in bryophytes: Purification and characterization of two L-asparaginase isoforms from Sphagnum fallax. Physiologia Plantarum. 1996;97(2):402-10.
31. Borek D, Podkowinski J, Kisiel A, Jaskolski M. Isolation and characterization of cDNA encoding L-asparaginase from Lupinus luteus. Plant Physiol. 1999;119:1568-70.
32. Bell TL, Adams MA. Ecophysiology of ectomycorrhizal fungi associated with Pinus spp. in low rainfall areas of Western Australia. Plant Ecology. 2004;171(1-2):35-52.
33. Bruneau L, Chapman R, Marsolais F. Co-occurrence of both L-asparaginase subtypes in Arabidopsis: At3g16150 encodes a K+-dependent L-asparaginase. Planta. 2006;224(3):668-79.
34. Gulati R, Saxena R, Gupta R. A rapid plate assay for screening l-asparaginase producing micro-organisms. Letters in applied microbiology. 1997;24(1):23-6.
35. Ghasemi Y, Ebrahiminezhad A, Rasoul-Amini S, Zarrini G, Ghoshoon MB, Raee MJ, et al. An Optimized Medium for Screening of L-Asparaginase production by< i> Escherichia coli</i>. American Journal of Biochemistry and Biotechnology. 2008;4(4):422.
36. Distasio JA, Niederman RA, Kafkewitz D, Goodman D. Purification and characterization of L-asparaginase with anti-lymphoma activity from Vibrio succinogenes. Journal of Biological Chemistry. 1976;251(22):6929-33.
37. Swain AL, Jaskólski M, Housset D, Rao J, Wlodawer A. Crystal structure of Escherichia coli L-asparaginase, an enzyme used in cancer therapy. Proceedings of the National Academy of Sciences. 1993;90(4):1474-8.
38. Aghaiypour K, Wlodawer A, Lubkowski J. Do bacterial l-asparaginases utilize a catalytic triad Thr-Tyr-Glu? Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology. 2001;1550(2):117-28.
39. Kozak M, Jurga S. A comparison between the crystal and solution structures of Escherichia coli asparaginase II. ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-. 2002;49(2):509-14.
40. Miller M, Rao J, Wlodawer A, Gribskov MR. A left-handed crossover involved in amidohydrolase catalysis: Crystal structure of Erwinia chrysanthemi L-asparaginase with bound L-aspartate. FEBS letters. 1993;328(3):275-9.
41. Palm GJ, Lubkowski J, Derst C, Schleper S, Röhm K-H, Wlodawer A. A covalently bound catalytic intermediate in< i> Escherichia coli</i> asparaginase: Crystal structure of a Thr-89-Val mutant. FEBS letters. 1996;390(2):211-6.
42. Michalska K, Jaskolski M. Structural aspects of L-asparaginases, their friends and relations. ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-. 2006;53(4):627.
43. Kiriyama Y, Kubota M, Takimoto T, Kitoh T, Tanizawa A, Akiyama Y, et al. Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase. Leukemia. 1989;3(4):294-7.
44. Prager MD, Bachynsky N. Asparagine synthetase in asparaginase resistant and susceptible mouse lymphomas. Biochemical and biophysical research communications. 1968;31(1):43-7.
45. Asselin BL, Ryan D, Frantz CN, Bernal SD, Leavitt P, Sallan SE, et al. In vitro and in vivo killing of acute lymphoblastic leukemia cells by L-asparaginase. Cancer research. 1989;49(15):4363-8.
46. Ueno T, Ohtawa K, Mitsui K, Kodera Y, Hiroto M, Matsushima A, et al. Cell cycle arrest and apoptosis of leukemia cells induced by L-asparaginase. Leukemia (08876924). 1997;11(11).
47. Shimizu T, Kubota M, Adachi S, Sano H, Kasai Y, Hashimoto H, et al. Pre-treatment of a human T-lymphoblastoid cell line with L-asparaginase reduces etoposide-induced DNA strand breakage and cytotoxicity. International journal of cancer. 1992;50(4):644-8.
48. HAN T, OHNUMA T. In vitro blastogenesis inhibited by Erwinia carotovora L-asparaginase. Nature. 1972;239(89):50-1.
49. Boyse EA, Old LJ, Campbell H, Mashburn LT. SUPPRESSION OF MURINE LEUKEMIAS BY L-ASPARAGINASE INCIDENCE OF SENSITIVITY AMONG LEUKEMIAS OF VARIOUS TYPES: COMPARATIVE INHIBITORY ACTIVITIES OF GUINEA PIG SERUM L-ASPARAGINASE AND ESCHERICHIA COLI L-ASPARAGINASE. The Journal of experimental medicine. 1967;125(1):17-31.
50. Crowther D. L-asparaginase and human malignant disease. 1971.

51. Howard JB, Carpenter FH. L-asparaginase from Erwinia carotovora substrate specificity and enzymatic properties. Journal of Biological Chemistry. 1972;247(4):1020-30.
52. Park Y, Abuchowski A, Davis S, Davis F. Pharmacology of Escherichia coli-L-asparaginase polyethylene glycol adduct. Anticancer research. 1980;1(6):373-6.
53. Berg SL, Balis FM, McCully CL, Godwin KS, Poplack DG. Pharmacokinetics of PEG-l-asparaginase and plasma and cerebrospinal fluidl-asparagine concentrations in the rhesus monkey. Cancer chemotherapy and pharmacology. 1993;32(4):310-4.
54. Schwartz MK, Lash ED, Oettgen HF, Tomao FA. L-asparaginase activity in plasma and other biological fluids. Cancer. 1970;25(2):244-52.
55. CAPIZZI RL, Bertino J, Skeel R, Creasey W, Zanes R, Olayon C, et al. L-asparaginase: clinical, biochemical, pharmacological, and immunological studies. Annals of internal medicine. 1971;74(6):893-901.
56. Haskell C, Canellos G. < i> l</i>-Asparaginase resistance in human leukemia—Asparagine synthetase. Biochemical pharmacology. 1969;18(10):2578-80.
57. Haskell C, Canellos G, Leventhal B, Carbone P, Block J, Serpick A, et al. L-asparaginase: therapeutic and toxic effects in patients with neoplastic disease. New England journal of medicine. 1969;281(19):1028-34.
58. urtzberg J, Asselin B, Poplack D, editors. PEG-L-asparaginase (PEG-ASP) pharmacology in pediatric patients with acute lymphoblastic leukemia (ALL). Proc Am Soc Clin Oncol; 1994.
59. Ho D, Yap H, Brown N, Benjamin R, Friereich E, Blumenschein G, et al. Clinical Pharmacology of Intramuscularly Administered l-Asparaginase. The Journal of Clinical Pharmacology. 1981;21(2):72-8.
60. Ho D, Whitecar JP, Luce JK, Frei E. L-asparagine requirement and the effect of L-asparaginase on the normal and leukemic human bone marrow. Cancer Research. 1970;30(2):466-72.
61. Worton KS, Kerbel RS, Andrulis IL. Hypomethylation and reactivation of the asparagine synthetase gene induced by L-asparaginase and ethyl methanesulfonate. Cancer research. 1991;51(3):985-9.
62. Broome J, Schwartz J. Differences in the production of L-asparagine in asparaginase-sensitive and resistant lymphoma cells. Biochimica et Biophysica Acta (BBA)-Nucleic Acids and Protein Synthesis. 1967;138(3):637-9.
63. Capizzi RL. Asparaginase revisited. Leukemia & lymphoma. 1993;10(S1):147-50.
64. Gentili D, Conter V, Rizzari C, Tschuemperlin B, Zucchetti M, Orlandoni D, et al. L-Asparagine depletion in plasma and cerebro-spinal fluid of children with acute lymphoblastic leukemia during subsequent exposures to Erwinia L-asparaginase. Annals of oncology. 1996;7(7):725-30.
65. Asselin BL, Whitin JC, Coppola DJ, Rupp IP, Sallan SE, Cohen HJ. Comparative pharmacokinetic studies of three asparaginase preparations. Journal of Clinical Oncology. 1993;11(9):1780-6.
66. Killander D, Dohlwitz A, Engstedt L, Franzen S, Gahrton G, Gullbring B, et al. Hypersensitive reactions and antibody formation during L-asparaginase treatment of children and adults with acute leukemia. Cancer. 1976;37(1):220-8.
67. Gallagher MP MR, Wilson R. l-Asparaginase a drugfor treatment of acute lymphoblastic leukemia. Essays in Biochemistry. 1989;24:1-40.
68. Oettgen HF, Stephenson PA, Schwartz MK, Leeper RD, Tallal L, Tan CC, et al. Toxicity of E. coli L-asparaginase in man. Cancer. 1970;25(2):253-78.
69. McEvoy G. American Hospital Formulary Service. American Society of Health-System Pharmacists. Inc, MD. 1993.
70. Jaffe N, Traggis D, Das L, Frauenberger G, Hann HW, Kim BS, et al. Favorable remission induction rate with twice weekly doses of L-asparaginase. Cancer research. 1973;33(1):1-4.
71. Bonno M, Kawasaki H, Hori H, Umemoto M, Komada Y, Sakurai M. Rapid desensitization for L-asparaginase hypersensitivity. The Journal of allergy and clinical immunology. 1998;101(4 Pt 1):571.
72. Rivera-Rodriguez GR, Lollo G, Montier T, Benoit JP, Passirani C, Alonso MJ, et al. < i> In vivo</i> evaluation of poly-l-asparagine nanocapsules as carriers for anti-cancer drug delivery. International journal of pharmaceutics. 2013;458(1):83-9.
73. Uren JR, Handschumacher RE. Enzyme therapy. Chemotherapy. 1977:457-87.
74. Abuchowski A, Van Es T, Palczuk N, McCoy J, Davis F. Treatment of L5178Y tumor-bearing BDF1 mice with a nonimmunogenic L-glutaminase-L-asparaginase. Cancer treatment reports. 1979;63(6):1127-32.
75. Uren JR, Ragin RC. Improvement in the therapeutic, immunological, and clearance

properties of Escherichia coli and Erwinia carotovora L-asparaginases by attachment of poly-DL-alanyl peptides. Cancer research. 1979;39(6 Part 1):1927-33.
76. Koerholz D, Brueck M, Nuernberger W, Juergens H, Goebel U, Wahn V. Chemical and immunological characteristics of four different L-asparaginase preparations. European journal of haematology. 1989;42(5):417-24.
77. Yoshimoto T, Nishimura H, Saito Y, Sakurai K, Kamisaki Y, Wada H, et al. Characterization of polyethylene glycol-modified L-asparaginase from Escherichia coli and its application to therapy of leukemia. Japanese journal of cancer research: Gann. 1986;77(12):1264-70.
78. Jürgens H, Schwamborn D, Körholz D, Wahn V, Göbel U. Klinische Erfahrungen mit polyäthylenglykol-gekoppelter E. coli-L-Asparaginase bei Patienten mit ALL-Mehrfachrezidiv. Klinische Pädiatrie. 1988;200(03):184-9.
79. Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, et al. Antibody against poly (ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. Cancer. 2007;110(1):103-11.
80. Davis F, Kazo G, Nucci M, Abuchowski A. Reduction of immunogenicity and extension of circulating half-life of peptides and proteins. Peptide and Protein Drug Delivery. 1991:831-64.
81. Nerkar D, Gangadharan M. Modification of L-asparaginase from Erwinia carotovora using human serum albumin. Molecular biotherapy. 1988;1(3):152-4.
82. Martins M, Jorge J, Cruz M. Acylation of L-asparaginase with total retention of enzymatic activity. Biochimie. 1990;72(9):671-5.
83. Gilbert HJ, Blazek R, Bullman HM, Minton NP. Cloning and expression of the Erwinia chrysanthemi asparaginase gene in Escherichia coli and Erwinia carotovora. Journal of general microbiology. 1986;132(1):151-60.
84. Moola ZB, Scawen M, Atkinson T, Nicholls D. Erwinia chrysanthemi L-asparaginase: epitope mapping and production of antigenically modified enzymes. Biochem J. 1994;302:921-7.
85. Kwon YM, Chung HS, Moon C, Yockman J, Park YJ, Gitlin SD, et al. L-Asparaginase encapsulated intact erythrocytes for treatment of acute lymphoblastic leukemia (ALL). Journal of Controlled Release. 2009;139(3):182-9.
86. Kurtzberg J, Friedman H, Asselin B, Schultz W, Krischer J, Scudiery D, et al., editors. The use of polyethylene glycol-conjugated L-asparaginase (PEG-ASP) in pediatric patients with prior hypersensitivity to native L-asparaginase. Proc Am Soc Clin Oncol; 1990.
87. Narta UK, Kanwar SS, Azmi W. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Critical reviews in oncology/hematology. 2007;61(3):208-21.
88. Ho D, Brown N, Yen A, Holmes R, Keating M, Abuchowski A, et al. Clinical pharmacology of polyethylene glycol-L-asparaginase. Drug metabolism and disposition. 1986;14(3):349-52.
89. Keating MJ, Holmes R, Lerner S, Ho DH. L-asparaginase and PEG asparaginase-past, present, and future. Leukemia & lymphoma. 1993;10(S1):153-7.
90. Vieira Pinheiro J, Müller H, Schwabe D, Gunkel M, Casimiro da Palma J, Henze G, et al. Drug monitoring of low-dose PEG-asparaginase (Oncaspartm) in children with relapsed acute lymphoblastic leukaemia. British journal of haematology. 2001;113(1):115-9.
91. Taylor CW, Dorr RT, Fanta P, Hersh EM, Salmon SE. A phase I and pharmacodynamic evaluation of polyethylene glycol-conjugated L-asparaginase in patients with advanced solid tumors. Cancer chemotherapy and pharmacology. 2001;47(1):83-8.
92. Ettinger LJ, Kurtzberg J, Voute P, Jurgens H, Halpern SL. An open-label, multicenter study of polyethylene glycol-L-asparaginase for the treatment of acute lymphoblastic leukemia. CANCER-PHILADELPHIA-. 1995;75:1176-.
93. Insert P. Oncaspar (pegaspargase) Rhone-Pooulenc Rorer. Collegeville, PA. 1994.
94. Silverman LB, Gelber RD, Dalton VK, Asselin BL, Barr RD, Clavell LA, et al. Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91-01. Blood. 2001;97(5):1211-8.
95. Domenech C, Thomas X, Chabaud S, Baruchel A, Gueyffier F, Mazingue F, et al. l-asparaginase loaded red blood cells in refractory or relapsing acute lymphoblastic leukaemia in children and adults: results of the GRASPALL 2005-01 randomized trial. British journal of haematology. 2011;153(1):58-65.
96. Yamaguchi M, Suzuki R, Kwong YL, Kim WS, Hasegawa Y, Izutsu K, et al. Phase I study of

dexamethasone, methotrexate, ifosfamide, l-asparaginase, and etoposide (SMILE) chemotherapy for advanced-stage, relapsed or refractory extranodal natural killer (NK)/T-cell lymphoma and leukemia. Cancer Science. 2008;99(5):1016-20.
97. Yamaguchi M, Kwong Y-L, Kim WS, Maeda Y, Hashimoto C, Suh C, et al. Phase II study of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer (NK)/T-cell lymphoma, nasal type: the NK-Cell Tumor Study Group study. Journal of Clinical Oncology. 2011;29(33):4410-6.
98. Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. Journal of General Microbiology. 1973;76(1):85-99.