Journal of
Medical Microbiology & Diagnosis

El-Ghonemy J Med Microb Diagn 2014, 4:1
DOI: 10.4172/2161-0703.1000173

Review Article Open Access

Microbial Amidases and their Industrial Applications: A Review

Dina H El-Ghonemy*

Department of Microbial Chemistry, National Research Centre, Dokki, Biotechnology and Genetic Engineering Division, Cairo, Egypt

Abstract

Among the pediatric cancer in developed countries, acute leukemia ¬constitutes the major part with affecting 30-45 per 1,000,000 children each year. The effect of treatment varies with differences in patients clinical, immunologic and genetic characteristics. Therefore, the search for efficient drugs to solve this problem is being continued worldwide. Although several kinds of treatments are available, enzyme therapy is equally effective. Enzymes have been used as drugs; likewise L-asparaginase and L-glutaminase had received much attention in recent years due to their anti-carcinogenic potential. These enzymes constitute one of the most biotechnologically and biomedically important group of therapeutic enzymes accounting for about 40% of the total worldwide enzyme sales. Various sources are found to be good producers of the enzymes: bacteria, fungi along with some of the plant and animal species. Food and Drug Administration and World Health Organization have approved L-asparaginase for the effective treatment of acute lymphoblastic leukemia and lymphsarcoma. L-asparaginase and L-glutaminase break down L-asparagine or L-glutamine into L-aspartic acid or L-glutamic acid, respectively, and ammonia. L-asparagine depletion results in nutritional deprivation, inhibition of protein synthesis, and subsequent apoptotic cell death in lymphoblasts. On the other hand, the ability of L-asparaginase to selectively hydrolyzes L-asparagine into L-aspartateis a potential way to reduce the amount of free L-asparagine in the starting materials of food production, thus reducing the imminent risk of generating a potential carcinogenic and neurotoxic acrylamide that formed from L-asparagine and reducing sugars in carbohydrate-containing foods (such as snacks and biscuits) when they are heated above 120oC. Therefore, the present review is an attempt to compile information on the sources, antino plastic action and industrial application of microbial amidases enzymes.

Keywords: Microorganisms; L-asparaginase; L-glutaminase; Anti-tumor activity; Industrial applications

Introduction

Enzymes are highly selective catalytic proteins which control and regulate all biochemical processes in the body. They are produced by living cells in order to accelerate both the rate and specificity of metabolic reactions. Enzymes are highly specific in their function because each enzyme is programmed to carry out one special task. Several million enzymes mediate chemical reactions occurring in a living system [1]. Microbial enzymes play a major role in the diagnosis, curing, biochemical investigation, and monitoring of many dreaded diseases. Microorganisms represent an excellent source of many therapeutic enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. The manufacture of enzymes for use as drugs is an important facet of today's pharmaceutical industry [2]. L-asparaginase (L-asparagine amidohydrolase; E.C. 3.5.1.1) has been proved to be a particularly promising enzyme in the treatment of acute lymphocytic leukemia (ALL) (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarbom and melanosarcoma [3]. In a study of almost 6.000 cases of acute lymphocytic leukemia about 60 % incidence of complete remission has been reported. Its action depends upon the fact that, tumor cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the normally non-essential amino acid, L-asparagine, therefore they are forced to extract it from body fluids [4].The action of L-asparaginase does not affect the functioning of normal cells, which are able to synthesize enough L-asparagine for their own requirements, but reduce the free exogenous concentration, and so induce a state of fatal starvation in the susceptible tumor cells [5]. Besides, L-asparaginase plays a central role in the amino acid metabolism and utilization. Where, in human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxalo acetate in the gluconeogenic pathway leading to glucose formation [6]. Moreover, this enzyme is also used in the food industry for the production of acrylamide-free food, as it aids to reduce the formation of acrylamide during frying of starchy foods at high temperature [7]. L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) has received also significant attention owing to its potential as an anticancer agent and a flavor enhancing agent in the food industry, as it increases the glutamic acid content of the food thereby imparting flavor. Its commercial importance demands not only the search for better yielding viable strains, but also economically viable bioprocesses for its large-scale production [8].

Historical Background

The pioneer observation of L-asparaginase as a potential antineoplastic agent was made by Clementi in 1922, who revealed the presence of high L-asparaginase activity in the serum of guinea pig only, whereas other mammals tested were found devoid of this enzyme. In 1953, Kidd described the regression of transplanted lymphomas in mice and rats by the administration of guinea pig serum. Broome in 1961 compared his finding of growth inhibition with the earliest observation by Clementi, and succeeded in concluding that the anti-lymphomatic activity in guinea pig sera was also due to L-asparaginase. Although Yellin and Wriston in 1966 succeeded in a

***Corresponding author:** Dina H El-Ghonemy, Department of Microbial Chemistry, National Research Centre, Dokki, Biotechnology and Genetic Engineering Division, Cairo, Egypt, Tel: 01062456850; E-mail: delghonamy@yahoo.com

Received September 28, 2014; **Accepted** December 13, 2014; **Published** December 15, 2014

Citation: Ghonemy DHE (2014) Microbial Amidases and their Industrial Applications: A Review . J Med Microb Diagn 4: 173. doi:10.4172/21610703.1000173

partial purification of two isoforms of L-asparaginase from the serum of guinea pig, other sources like microbes were recommended, since the extraction of L-asparaginase from the guinea pig serum in sufficient amounts was difficult. In this concern, many investigators reported the purification of *Escherichia coli* L-asparaginase, and demonstrated the tumoricidal activity of this purified enzyme which was similar to that of guinea pig sera [9]. Oettgen et al. [10] were the first to show the efficacy of L-asparaginase in humans with leukemia. Furthermore, in April 2002, an application for L-asparaginase was found in food technology, when Swedish researchers sparked the world's attention with their preliminary findings of acrylamide in some fried and baked foods, most notably potato chips and French fries [11]. The ability of L-asparaginase to selectively hydrolyze asparagine to aspartate and ammonia was a potential way to reduce the amount of free asparagine in the starting materials of food production, thus reducing the imminent risk of generating a potentially carcinogenic and neurotoxic acrylamide [12]. In parallel, the anticancer activity of L-glutaminase was firstly proposed by [13]. On the other hand, Yamamoto and Hirooka in 1974 isolated two strains of L-glutaminase producing *Aspergillus sojae* from soya mash. They observed that this L-glutaminase plays a vital role in improving the taste of sauce. This taste was due to the presence of L-glutamate which is a well-known flavor enhancing amino acid and imparts a savory taste (umami).

Antineoplastic action

L-asparaginase catalysis the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl enzyme as intermediate (Figure 1) [14]. The mechanism of action of L-asparaginase has been elucidated by Broome in [15] (Figure 2). Where, following the administration of L-asparaginase, the non-essential amino acid asparagine is hydrolyzed into aspartic acid and ammonia, prevents protein synthesis of neoplasms. It produces cell death through activation of apoptosis. The substrate L-asparagine, essential for the growth of tumor cells, can be reduced by injecting the enzyme L-asparaginase and making the required substrate (L-asparagine) unavailable to tumor proliferation [16,17]

In most human normal cells, deficiency of asparagine can be compensated by alternative synthesis pathway through which asparagine is produced from aspartic acid and glutamine by asparagines synthethase (Nakamura et al.). [18] While malignant cells (that respond to treatment) are probably unable to ensure their own asparagine supply due to the deficiency in asparagines synthetase activity which is lower than that found in normal cells, and thus depends on the extracellular availability of this amino acid [19]. The asparagine deficiency rapidly impairs the protein synthesis [20] leading to a delayed inhibition in DNA and RNA synthesis and hence to the impairment of cellular function and cell death (Figure 2).

The use of glutaminase to deplete glutamine in tumor-bearing hosts offers an attractive method for attacking cancer cells [21]. High rate of glutamine consumption is also a characteristic nature of some types of cancerous cells [22]. Inhibition of the tumor cell uptake of glutamine is one of the possible ways to stop the growth and this is best accomplished by the use of L-glutaminase, which breaks down L-glutamine. Compared with normal tissues, some neoplasms have been shown to operate at a marginal level of glutamine availability because of decreased synthesis and stepped-up utilization. This in fact, results in a selective starvation of the tumor cells because unlike normal cells, they lack properly functioning glutamine biosynthetic machinery [23].

Occurrence and distribution

Amidases are widely distributed in plants, animals, rodents and microorganisms including bacteria, fungi, actinomycetes [24-26]. However, the inability of the plant and animal amidases to meet current world demands has led to an increased interest in microorganisms which represent an excellent source of enzymes [8]. Most of the microbial L-asparaginase and L-glutaminase are intracellular in nature except few, which are secreted outside the cells [27].

Microbial amidases

Microorganisms produce L-asparaginase and L-glutaminase either constitutively or after induction [28]. The production titer values of these enzymes are influenced by microbial strain and fermentation conditions [26]. This is because microbial metabolism is highly interactive and influenced by environmental, fermentation and nutritional factors at the interactive level [29]

Bacterial amidases

Bacterial amidases are enzymes of high therapeutic value for use in certain kinds of cancer therapies, mainly in treatment of acute lymphoblastic leukemia [30]. Not all of these bacterial enzymes have anti-tumor properties; the variation in anti-tumor activity has been related to the affinity of the enzyme for its substrate and the clearance rate of the particular types of enzymes [9]. Commercially used enzymes are obtained from *E. coli* and *Erwinia carotovora*. They have been used successfully in the treatment of leukemia for the last 40 years [31,32]. A large proportion of strains of *Pseudomonas* species showed L-asparaginase and L-glutaminase simultaneously. Most members of the *Enterobacteriaceae* are active for L-asparaginase but not so active for L-glutaminase [33]. While Roberts et al. [21] isolated *Achromobacteraceae* from the soil samples and observed that this family has L-glutaminase and L-asparaginase activity in a ratio of 1.2:1. There are also many reports on the production of the intracellular L-asparaginase as observed from *E. aroideae* [34] *E.carotovora* [35] *Serratia marcescens* [36] ,*Vibrio succinogenes* [37], *E. coli* [38], *Pseudomonas*7A (Jakob et al., 1996) [39] and *P.fluorescens* [40].

Yeast amidases

Little is known about the properties of yeast L-asparaginase and L-glutaminase. The productionof L-asparaginase from *Saccharomyces cerevisiae* was found to be constitutive both in periplasmic and cytoplasmic fractions [41,42]. Extracellular formation of L-asparaginase by *Candida utilis* and *Rhodotorula rosa* was reported by [43]. While [33] observed L-asparaginase in culture filtrates of *Hansenula jadini,Rhodotorula rubra, Cryptococcus albidus, Sporobolomyces*

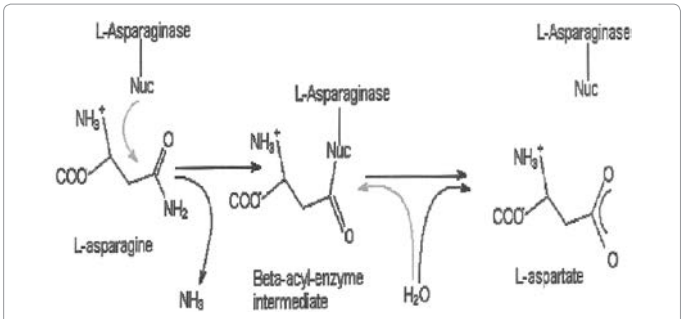

Figure 1: Schematic illustration of the reaction mechanism of L-asparaginase

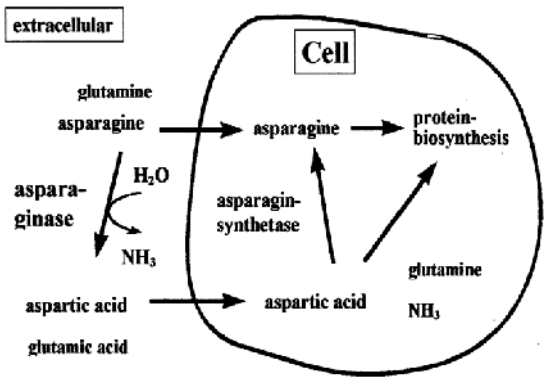

Figure 2: Schematic illustration of the antineoplastic action of L-asparaginase

roseus and *Candida utilis*. Moreover, they investigated the production of a single L-glutaminase from many strains of *Candida scottii* and *Cryptococcus albidus*. Foda et al. [44] isolated *Pichia polymorpha* with high potentialities for L-glutaminase and L-asparaginase formation from the Egyptian soils.

Actinomycetes amidases

Actinomycetes serve as a good source for L-asparaginase. *Streptomyces longsporusflavus*, *S. albidoflavus* and a marine *Streptomyces* sp. PDK2 are capable of producing detectable amounts of L-asparaginase [27,45]. Sivakumar et al. [46] isolated different actinomycetes from skin, gills and gut contents of estuarine fish, *Chanos chanos* using Kuster`s agar medium. Out of the 20strains tested, *Streptomyces rimosus* showed L-glutaminase activity.

Fungal amidases

Aspergillus, *Penicillium*, and *Fusarium*, are commonly reported in scientific literature to produce L-asparaginase and L-glutaminase [47-50]. Mohapatra et al. [51] isolated the fungus *Mucor* sp. from the marine sponge *Spirastrella* sp. which produces the extracellular L-asparaginase. Yamamoto and Hirooka [52] isolated two strains of *Aspergillus sojae* from soya mash capable of producing L-glutaminases sufficiently. Sabu et al. [53] reported L-glutaminase production by *Beauveria* sp. using polystyrene as solid support under solid state fermentation. Thammarongtham et al. [54] purified L-glutaminase from *Aspergillus oryzae* by using ammonium sulfate precipitation, then applied to Q-Sepharose ion exchange column and finally chromatographed on Sephacryl S-200 HR gel filtration column. L-glutaminaseproduction by *Aspergillus wintii* KGSD4 isolated from soil of Bangalore was investigated by Siddalingeshwara et al. [14]. Pallem et al. [55] also reported an extracellular L-glutaminase enzyme by *Trichoderma koningii* in solid-state fermentation. In 2012 Elshafei et al. [49] purified an intracellular glutaminase-free-L-asparaginase from *Penicillium brevicompactum* NRC 829 to homogeneity with an apparent molecular mass (Mr) of 94 kDa. The purified enzyme was 151.12 fold with a final specific activity of 574.24 IU/mg protein and about 40 % yield recovery. In addition, they reported that, the purified L-asparaginase inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2), with IC_{50}value of 43.3µg/ml. Nagarajan et al. [56] identified several endophytes capable of producing glutaminase free L-asparaginase. Among all the screened endophytes, they found that WS/ *Alternaria* sp. 2, produced maximum glutaminase free L-asparaginase. They also studied the optimal concentrations of glucose and L-asparagine for maximum enzyme production which found to be at 10 and 1 g/L, respectively.

Applications of Microbial Amidases

Therapeutic applications

The Food and Drug Administration (FDA) and World Health Organization (WHO) have approved L-asparaginase for the effective treatment of acute lymphoblastic leukemia (ALL) and lymphsarcoma. L-asparaginase activity was widely reported in plants, animals and microorganisms, but only the asparaginase from *E. coli* and *Erwiniachrysanthemi* have been produced on industrial scale. Although both drugs have identical mechanism of action and toxicities, their pharmacokinetic properties are different, and patients allergic to one drug are frequently resistant to the other [57]. ELSAPAR, ONCASPAR, ERWINASE and KIDROLASE are the brand names of L-asparaginase [58,59]. Pegaspargase is a modified version of the enzyme L-asparaginase. Toproduce pegaspargase, L-asparaginase is covalently conjugated to units of monomethoxy polyethylene glycol (PEG), forming the active gradient PEG-L-asparaginase. PEG-asparaginase was found to be a useful alternative in patients with prior clinical hypersensitivity to native *E. coli* asparaginase. PEG- asparaginase reduces the immunogenicity of the protein, increases its stability in plasma and is suitable for use in heavily pre-treated patients [60]. The recommended dose of pegaspargase is 2.5 IU/m^2 Intramuscularly (IM) or Intravenously (IV), and should be administered for every 14 days. PEG-asparaginase has a longer half-life (app. 6 days) and shows a slower clearance than *Erwinia* or *E. coli* asparaginase. Therefore, PEG asparaginase can be administered at lower doses and at longer intervals [61]. *Erwinia* asparaginase is used in combination with other chemotherapeutic agents for the treatment of acute lymphoblastic leukemia in patients who have hypersensitivity to the L-asparaginase drug derived from *E. coli* [57]. Therefore, the clinical utility of L-asparaginase is often limited by three factors. First, the side effects associated with L-asparaginase administration, including immunosuppression and pancreatitis [62]. Second, about 10% of successfully treated patients suffer a relapse with the appearance of tumors that are resistant to further L-asparaginase therapy [63]. Lastly, prolonged treatment with L-asparaginase improves the growth of resistant tumors and increases the metastatic activity [64]. A parallel interest in L-glutaminase has arisen from demonstrations that microbial glutaminase also exhibits antitumor activity. A glutaminase–asparaginase enzyme from *Achromobacter* has shown anti leukemic effects in patients with acute lymphoblastic leukemia and acute myelois leukemia in a preliminary clinical trial [65]. Moreover, one of the most promising therapeutic applications ever proposed for the L-glutaminase is in treatment of HIV [66].

Applications of amidases in food industry

Glutamic acid and aspartic acid have been well known as important amino acids contributing not only fine taste, "*Umami*" and sharp taste "Sour", but also nutritional effects to food. The pleasant and palatable tastes of oriental fermented foods like soy sauce is considered to be related to the content of L-glutamic acid [67] which accumulates following the hydrolysis of a protein component catalyzed by proteolytic enzymes, including L-glutaminase, protease and peptidases. In addition, aspartic acid, a product of asparaginase-glutaminase catalyzed reaction, is converted by aspartic acid decarboxylase into alanine, a decisive amino acid component of soy sauce. The most common use of asparaginase is as a processing aid in the manufacture of food [68], which are marketed under the brand names Acrylaway and Prevent ASe. The ability of L-asparaginases to selectively hydrolyze asparagine into L-aspartate and ammonia is a potential way to reduce the amount of free L-asparagine in the starting materials of food production, thus

reducing the imminent risk of generating a potential carcinogenic and neurotoxic compound called acrylamide that formed from L-asparagine and reducing sugars in carbohydrate-containing foods (such as snacks and biscuits) when they are heated above 120°C [12]. Novozymes A/S Denmark (submitted by Novozymes Australia Pty Ltd) is now seeking an approval for recombinant L-asparaginase as food processing aid [69,70]. Treatment of raw products with asparaginase before thermal treatment resulted in a 96-99 % drop of acrylamide formation [71,72]. Therefore, it is not surprising that L-glutaminase is researched as a catalyst for the production of fermented condiments such as Japanese soy sauce [73].

Analytical applications

Analyses of L-glutamine and glutamate levels of the body fluids are important in clinical diagnostics and health monitoring. L-Glutaminase for biosensor application was investigated by the Kikkoman Corporation, Japan [74]. Botre et al. [75] used L-glutaminase based biosensor for determination of glutamine and glutamate in pharmaceutical formulations. The enzyme has also been widely employed in hybridoma culture media [76] and the monitoring of glutamine and glutamate levels in mammalian cell culture media by flow injection analysis using biosensors [77,74].

Manufacture of fine-chemicals

The most important emerging application of L-glutaminase in industry comes from its use in the manufacture of γ –glutamyl alkamides. L-γ-glutamyl alkamides are prepared by γ-glutamyl transfer from a donor such as glutamine or glutathione to a glutamyl acceptor like ethylamine, methyl amine or glycyl glycine. Theanine (γ-l-glutamyl ethylamide) is one of the major components of amino acids in Japanese green tea, and unique as a taste-enhancing amino acid of infused green tea. Recently, increasing attention has been directed towards the physiological roles of theanine, especially in a clinical point of view because of their ability to suppress stimulation by caffeine, to improve effects of antitumor agents, and their role as antihypertensive agents. Tachiki et al. [78] used L-glutaminase from *P. nitroreducens* for the production of γ-glutamyl-methylamide in addition to threonine by using methylamine as γ-glutamyl acceptor [5].

Toxicity of amidases: Hypersensitivity

Unfortunately, asparaginase and glutaminase can cause an allergic reaction leading to inactivation of the drug or silent inactivation. The route of administration determines the clinical symptoms. Two possible mechanisms have been proposed for L-asparaginase resistance [63]. The first is related to an increase in asparagine synthase levels, which has been found in the blasts cells of patients with acute lymphoblastic leukemia clinically resistant to the drug [64]. Another mechanism appears to be the production of anti-asparaginase antibodies in the host cells, which neutralize L-asparaginases impeding their enzymatic activity (Chakrabarti and Schuster). Some studies have shown that the incidence of hypersensitivity to asparaginase is similar between age groups; others suggested that infants and younger patients develop antibody and hypersensitivity reactions less frequently than teenagers and adult patients [79]. Antibodies produced in response to asparaginase do not always lead to clinical hypersensitivity instead cause rapid inactivation of the asparaginase, resulting in Sub optimal asparagines depletion. This is commonly referred to as silent hypersensitivity or silent inactivation [80]. While in adult patients, liver toxicities with elevated liver enzymes and increased bilirubin are a frequent clinical problem [81]. However, in the case of allergic reactions to PEG-asparaginase, *Erwinia* asparaginase is given instead. *Erwinia* asparaginase is given three times per week. The different dose schedules for native *E. coli* asparaginase, PEG-asparaginase and *Erwinia* asparaginase are based on differences in the pharmacokinetics of the three products [82].

Cost of drug

Asparaginase is an expensive drug, but important in childhood acute lymphoblastic leukemia. Asparaginase costs are mainly determined by the percentage of patients who are allergic and require a switch to *Erwinia* asparaginase. Tong et al. [83] reported that, the total costs of the intensification course of 30 weeks were $57,893 in patients without PEG-asparaginase allergy (n=64). While, the costs were significantly higher ($113,558) in case of allergy (n=20) necessitating a switch to *Erwinia* asparaginase. They also found that using native *E. coli* asparaginase in intensification showed that the costs of PEG-asparaginase were equal to those of native *E. coli* asparaginase. However, PEG-asparaginase is preferred over native *E. coli* asparaginase, because it is administered less frequently, with less day care visits. PEG-asparaginase is less immunogenic than native *E. coli* asparaginase and is not more expensive [83].

Conclusion

Enzymes are in great demand for use as therapeutic agents against many dreadful diseases. L-asparaginase and L-glutaminase are oncolytic enzymes involved in the deamination of L-asparagine and L-glutamine, respectively. Various sources are found to be good producers of the enzymes: bacteria, fungi along with some of the plant and animal species. L-asparaginase and L-glutaminase are very promising agents in the treatment of acute lymphoblastic leukemia and other kinds of cancer. Whereas, normal tissues can synthesize L-asparagine and L-glutamine while cancerous cells (especially malignant and carcinoma cell) require external source of these amino acids for their growth and multiplication. Thus, in the presence of these enzymes, the tumor cells failure to survive. Therefore, L-asparaginase and L-glutaminase can be used as chemotherapeutic agents for the treatment of ALL (mainly in children) as potent antitumor or antileukematic drugs. Apart from their role in the treatment of cancer, L-asparaginase and L-glutaminase also used in food industry; L-asparaginase used to reduce the formation of acrylamide, a carcinogen found in starchy food products, while L-glutaminase used to increase the glutamic acid content of the food thereby imparting flavor.

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