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REVIEW ARTICLE

Therapeutic L-asparaginase: upstream, downstream and beyond

André Moreni Lopes[1], Laura de Oliveira-Nascimento[2], Artur Ribeiro[3], Carlos Abrunhosa Tairum Jr[4], Carlos Alexandre Breyer[4], Marcos Antonio de Oliveira[4], Gisele Monteiro[1], Cristina Maria de Souza-Motta[5], Pérola de Oliveira Magalhães[6], Jorge Gonzalo Farías Avendaño[7], Artur Manuel Cavaco-Paulo[3], Priscila Gava Mazzola[8], Carlota de Oliveira Rangel-Yagui[1], Lara Durães Sette[9], Attilio Converti[10], and Adalberto Pessoa[1]

[1]Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil, [2]Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas – UNICAMP, Campinas, Brazil, [3]Centre of Biological Engineering (CEB), School of Engineering, University of Minho, Braga, Portugal, [4]Biosciences Institute, Coastal Campus, São Paulo State University – UNESP, São Vicente, Brazil, [5]Department of Mycology, Federal University of Pernambuco, Recife, Brazil, [6]Department of Pharmacy, School of Health Sciences, University of Brasilia – UnB, Brasilia, Brazil, [7]Department of Chemical Engineering, Faculty of Engineering and Science, University of La Frontera, Temuco, Chile, [8]Faculty of Pharmaceutical Sciences, University of Campinas – UNICAMP, Campinas, Brazil, [9]Department of Biochemistry and Microbiology, Institute of Biosciences, São Paulo State University – UNESP, Rio Claro, Brazil, and [10]Department of Civil, Chemical and Environmental Engineering, University of Genoa, Genoa, Italy

Abstract

L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) is an enzyme clinically accepted as an antitumor agent to treat acute lymphoblastic leukemia and lymphosarcoma. It catalyzes L-asparagine (Asn) hydrolysis to L-aspartate and ammonia, and Asn effective depletion results in cytotoxicity to leukemic cells. Microbial L-asparaginase (ASNase) production has attracted considerable attention owing to its cost effectiveness and eco-friendliness. The focus of this review is to provide a thorough review on microbial ASNase production, with special emphasis to microbial producers, conditions of enzyme production, protein engineering, downstream processes, biochemical characteristics, enzyme stability, bioavailability, toxicity and allergy potential. Some issues are also highlighted that will have to be addressed to achieve better therapeutic results and less side effects of ASNase use in cancer treatment: (a) search for new sources of this enzyme to increase its availability as a drug; (b) production of new ASNases with improved pharmacodynamics, pharmacokinetics and toxicological profiles, and (c) improvement of ASNase production by recombinant microorganisms. In this regard, rational protein engineering, directed mutagenesis, metabolic flux analysis and optimization of purification protocols are expected to play a paramount role in the near future.

Keywords

Acute lymphoblastic leukemia, antineoplastic activity, biopharmaceutical, L-asparaginase, microbial L-asparaginase production

History

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Introduction

L-asparaginase (ASNase) is an enzymatic drug and an essential component of the combination chemotherapy against diseases such as acute lymphoblastic leukemia (ALL), lymphosarcoma, Hodgkin's disease, acute myelogenous leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, reticulosarcoma and melanosarcoma (Avramis & Tiwari, 2006; Kumar et al., 2014; Pui & Evans, 2006; Verna et al., 2007). This drug depletes L-asparagine (Asn) in blood, blocking protein synthesis and inhibiting DNA and RNA synthesis in cancer cells. As a result, cell functions are impaired resulting in apoptosis (Bussolati et al., 1995).

Address for correspondence: André M. Lopes, Department of Bioengineering, University of California Los Angeles – UCLA, 420 Westwood Plaza - 5121J Engineering V, P.O. Box 951600, CA 90095-1600. E-mail: andreml@usp.br

Normal cells, however, are able to synthesize Asn and are less affected by its depletion by treatment with ASNase. Nonetheless, when used for long-term treatment, it may cause hypersensitivity leading to allergic reactions such as skin rashes, respiratory disorders, low blood pressure, sweating and loss of consciousness (Sarquis et al., 2004) as well as anaphylaxis (Verna et al., 2007). Different ASNase preparations from *Escherichia coli* [native (EcA) and PEGylated form] or *Erwinia chrysanthemi* [native form (ErA)] are available on the market (Tong et al., 2014). Additionally, a PEGylated recombinant *E. chrysanthemi*-derived ASNase is currently in Phase I clinical trials (http://www.clinicaltrials.gov/show/NCT01551524).

Mashburn & Wriston (1963) proved that purified *E. coli* ASNase exerted the same therapeutic effect as guinea pig serum (Jain et al., 2012), with the advantage of easier production at lower cost (Schalk & Lavie, 2014). Asn has also shown to be a fundamental nutritional requirement for the

in vitro growth of both Walker carcinosarcoma 256 and leukemic mice cells.

In recent years, there has been increased interest in ASNase use to treat ALL in adults, particularly young adults (Rytting, 2012). Out of the 4000 ALL cases diagnosed yearly in the USA, approximately two-thirds are children and adolescents, making ALL the most common cancer among this age group (Apostolidou et al., 2007; Pui & Evans, 2006). Long-term improvement in children was reported to be around 80%, and the overall survival rate 90%, while in adults these figures were 38% and 50%, respectively (De Bont et al., 2004; Pui & Howard, 2008). In recent decades, there has been considerable progress in leukemia treatment. However, because of issues related to the access to treatment, significant differences in survival are reported among populations. The five-year survival rate is 43% in the USA and Western Europe, 25% in Japan, 24% in South America, 19% in India, 15% in Thailand, and 14% in sub-Saharan Africa. In areas with access to treatment, the five-year survival rate in children can reach 80% (Datasus, 2013; INCA, 2014).

ASNase is widely distributed in nature, being found not only in microorganisms, but also in plants and tissues (liver, pancreas, brain, ovary or testes, kidneys, spleen and lungs) of several animals like fishes, mammals and birds. However, microbes are a better source than animals or plants, considering their ability to grow easily on rather simple and inexpensive substrates. Furthermore, they offer easy optimization of culture conditions for enzyme bulk production, easy genetic modification to increase the yield, economically-viable extraction and purification, good stability and consistency (Thakur et al., 2014), coupled with the *ex situ* preservation.

Taking into account this scenario, the aim of this review is to provide a thorough review of microbial ASNase production. More specifically, it focuses on microorganisms, conditions for enzyme production by either submerged or solid-state fermentation, applications, biochemical characteristics and manipulation, and downstream processes.

Upstream processing – ASNase production

Bacterial fermentation

Although several species of bacteria are reported to produce ASNase, *E. coli* and *E. chrysanthemi* are at present the main microbial agents for industrial-scale production. ASNases from these microorganisms have similar mode of action, antineoplastic activity and toxicity, but are serologically and biochemically distinct and have different pharmacokinetic profiles (Kumar & Sobha, 2012).

ASNase has mainly been produced by bacterial or fungal submerged fermentation (SF). Experimental evidence has shown that this production is greatly influenced by various factors such as type and concentration of carbon and nitrogen sources, pH, temperature, fermentation time, aeration and mainly the microbial agent (Basha et al., 2009; Gurunathan & Sahadevan, 2012; Zia et al., 2013).

A comprehensive review on the main aspects of ASNase production by bacterial SF was published by Kumar & Sobha (2012), to which the reader is invited to refer. Therefore, a simple list of the most significant bacteria able to extracellularly express ASNase is provided (Table 1) (Abdel-Fatah, 1997; Abdel-Fatah et al., 1998; Alegre & Minim, 1993; Amena et al., 2010; Basha et al., 2009; Fisher & Wray, 2002; Geckil & Gencer, 2004; Geckil et al., 2006; Gladilina et al., 2009; Gunasekaran et al., 1995; Kenari et al., 2011; Koshi et al., 1997; Kotzia & Labrou, 2005; Kotzia & Labrou, 2007; Kumar et al., 2010; Lebedeva & Berezov, 1997; Lubkowski et al., 1996; Mahajan et al., 2012; Manna et al., 1995; Narayana et al., 2008; Nawaz et al., 1998; Pinheiro et al., 2001; Prakasham et al., 2007; Pritsa & Kyriakidis, 2001; Ramaiah & Chandramohan, 1992; Sidoruk et al., 2011; Sinha et al., 1991; Thenmozhi et al., 2011).

Table 1. Main bacteria employed in SF for L-asparaginase production.

Taxon	Reference	Taxon	Reference
Actinomycetes (Marine)	(Basha et al. 2009)	*Pseudomonas* spp.	
Bacillus spp.		*P. aeruginosa*	(Geckil et al. 2006)
B. cereus	(Thenmozhi et al. 2011)	*P. aurantiaca*	(Lebedeva & Berezov 1997)
B. licheniformis	(Mahajan et al. 2012)	*P. stutzeri*	(Manna et al. 1995)
B. subtilis	(Fisher & Wray 2002)	*Staphylococcus* spp.	(Prakasham et al. 2007)
Enterobacter spp.		*Streptomyces* spp.	
E. aerogenes	(Geckil & Gencer 2004)	*S. albidoflavus*	(Narayana et al. 2008)
E. cloacae	(Nawaz et al. 1998)	*S. gulbargensis*	(Amena et al. 2010)
Erwinia spp.		*S. longsporusflavus*	(Abdel-Fatah 1997)
E. aroideae	(Alegre & Minim 1993)	*S. phaeochromogenes*	(Abdel-Fatah et al. 1998)
E. carotovora	(Kotzia & Labrou 2005)	*S. plicatus*	(Koshi et al. 1997)
E. chrysanthemi	(Kotzia & Labrou 2007)	*Thermus* spp.	
Escherichia coli	(Kenari et al. 2011)	*T. thermophilus*	(Pritsa & Kyriakidis 2001)
Helicobacter spp.		*Vibrio* spp.	
H. pylori	(Gladilina et al. 2009)	*V. fisheri*	(Ramaiah & Chandramohan 1992)
Nocardia spp.		*V. hawveyi*	
N. asteroides	(Gunasekaran et al. 1995)	*V. proteus*	(Sinha et al. 1991)
Pectobacterium spp.		*Wolinella* spp.	
P. carotovorum	(Kumar et al. 2010)	*W. succinogenes*	(Lubkowski et al. 1996)
Photobacterium spp.		*Yersinia* spp.	
P. leiognathi	(Ramaiah & Chandramohan 1992)	*Y. pseudotuberculosis*	(Sidoruk et al. 2011)
P. phoshoreum		*Zymomonas* spp.	
		Z. mobilis	(Pinheiro et al. 2001)

Members of the Enterobacteriaceae family are undoubtedly the best ASNase producers among the eubacteria. The addition of 6% *n*-dodecane under dissolved oxygen levels above 80% increased the *E. coli* cell concentration by 12.7% and ASNase activity (up to 60.80 IU/mL) by 21% (Wei & Liu, 1998). Among the other Enterobacteriaceae, when 10 g/L lactose or cheese whey was used as the carbon source for bench-scale batch cultivation of *Erwinia aroidae*, the addition of Asn and yeast extract or tryptone remarkably stimulated ASNase production (Alegre & Minim, 1993; Minim & Alegre, 1992). Also, the Gram-negative, rod-shaped bacterium *Enterobacter cloacae* was able to successfully utilize Asn either as the sole carbon and nitrogen source or in combination with L-fructose, D-galactose, sucrose or maltose and expressed ASNase intracellularly (Nawaz et al., 1998). ASNase production was also reported in *Enterobacter aerogenes* cultured on different carbon sources such as glucose, lactose, mannitol and glycerol (Geckil & Gencer, 2004; Geckil et al., 2005), but its activity was low (0.60 U/mL).

Another eubacterium able to express high levels of ASNase is *Pectobacterium carotovorum* MTIC 1428 (Kumar et al., 2009); when grown under optimized conditions on a medium containing glucose, yeast extract, peptone and Asn, it exhibited a maximum activity of 15.39 U/mL (specific activity of 27.88 U/mg) after only 12 h along with the highest volumetric productivity (1282 U/mL h) reported in the literature, to the best of our knowledge.

Although reports on ASNase production by filamentous bacteria are quite scarce, some of them, especially marine actinomycetes, were shown to be effective producers of this enzyme, among which is the thermophilic soil isolate S3 that displayed a ASNase activity as high as 49.20 U/mL at pH 7.5 and 50 °C (Basha et al., 2009). These results open a new scenario in the search for more heat-resistant ASNases from thermophilic bacteria.

In recent years, solid-state fermentation (SSF) has emerged as a valid alternative to SF for the production of extracellular enzymes (Venil & Lakshmanaperumalsamy, 2009), because it allows employing agroindustrial wastes as nutrient sources and holds potential for the production of secondary metabolites, especially in developing countries (Sangeetha et al., 2004). In comparison with SF, only a few reports are available on SSF for ASNase production, mainly using agroindustrial wastes, among which are soy bean meal (Abdel-Fattah & Olama, 2002; Basha et al., 2009; El-Bessoumy et al., 2004), rice bran (Venil & Lakshmanaperumalsamy, 2009), gram husk and coconut (Hymavathi et al., 2009). The most significant results found in the literature on SSF for ASNase production by bacteria are summarized in Table 2 (Abdel-Fattah & Olama, 2002; El-Bessoumy et al., 2004; Ghosh et al., 2013; Hymavathi et al., 2009; Venil & Lakshmanaperumalsamy, 2009; Vuddaraju et al., 2010).

To provide only the most significant examples, optimization of SSF for ASNase production by *Pseudomonas aeruginosa* and *Serratia marcescens* has been reported by several authors. El-Bessoumy et al., (2004) obtained, under optimal conditions, ASNase activity of 165.1 IU/mL (specific activity of 17.90 IU/mg) after 96 h of fermentation by *P. aeruginosa* 50071 in a medium with 40% moisture content containing 22 g/L soy bean meal, at pH 7.4 and 37 °C. When 3.11% (w/v) casein hydrolyzate and 3.68% (w/v) corn steep liquor were added as supplementary nutrients to the same medium (20 g/L soy bean meal), but with higher moisture content (50%), this strain exhibited, under comparable conditions (pH 7.0; 37 °C; 96 h), 38% lower specific activity (Abdel-Fattah & Olama, 2002), which confirms the influence of both environmental and nutritional conditions on SSF performance.

Fungal fermentation

Because all available ASNase therapeutic preparations are from prokaryotic sources, anaphylactic and other immunological side effects as well as enzyme inactivation are frequent events. In this sense, the search for ASNase sources

Table 2. Summary of fermentation conditions and results of L-asparaginase production by bacteria in SSF.

Microorganism	Substrates/conditions	L-asparaginase production	Reference
P. aeruginosa 50071	Soy bean meal 20 g/L; moisture content 50%; casein hydrolysate 3.11% (w/v); corn steep liquor 3.68% (w/v); pH 7.0; 37 °C; 96 h	11.15 IU/mg of proteins	(Abdel-Fattah & Olama 2002)
	Soy bean meal 22 g/L; moisture content 40%; pH 7.4; 37 °C; 96 h.	17.90 IU/mg of proteins; 165.1 IU/mL of crude extract	(El-Bessoumy et al. 2004)
Bacillus circulans MTCC 8574	Red gram husk; moisture content 99.5%; glucose 0.234% (w/w); Asn 0.248% (w/w); pH 7.0; 36.3 °C; 24 h.	2322 U/gds	(Hymavathi et al. 2009)
S. marcescens SB08	Rice bran 10 g; moisture content 40%; Asn 0.01%; yeast extract 0.5%; pH 7.0; 30 °C; 36 h.	79.84 U/gds	(Venil & Lakshmanaperumalsamy 2009)
S. marcescens NCIM 2919	Sesame oil cake 40 g/L; moisture content 68.64%; glucose 3.1% (w/w); $NaNO_3$ 1.01% (w/w); pH 7.0–7.5; 30 °C; 48 h.	110.8 U/gds	(Vuddaraju et al. 2010)
	Coconut oil cake 6 g; moisture content 40%; pH 6.0; 37 °C.	3.87 U/gds	(Ghosh et al. 2013)

Units are different because studies report values expressed in different units. IU, international unit; gds, grams of dried substrate.

Table 3. Main yeasts and filamentous fungi employed for L-asparaginase production in SF.

Taxon	References	Taxon	Reference
Aspergillus spp.		*Fusarium* spp.	
A. niger	(Zia et al. 2013, Alhussaini 2013)	*F. moniliforme*	(Tippani & Sivadevuni 2012)
A. tamari	(Sarquis et al. 2004)	*F. semitectum*	(Alhussaini 2013)
A. terreus	(Gurunathan & Sahadevan 2012)	*Paecilomyces* spp.	(Gupta et al. 2009)
A. flavus	(Alhussaini 2013)	*Penicillium* spp.	
Bipolaris spp.	(Lapmak et al. 2010)	*P. chrysogenum*	(Alhussaini 2013)
Candida spp.		*P. crustosum*	
C. utilis	(Kil et al. 1995)	*P. digitatum*	(Shrivastava et al. 2012)
Cladosporium spp.	(Kumar & Manonmani 2013)	*P. olsonii*	(Alhussaini 2013)
Cylindrocarpon spp.		*Rhodosporidium* spp.	
C. obtusisporum	(Raha et al. 1990)	*R. toruloides*	(Ramakrishnan & Joseph 1996)

from eukaryotic microorganisms can lead to enzymes with lower side effects to humans (Shrivastava et al., 2012). To achieve this purpose, the use of screening techniques is an important step of any prospecting process, which can be made based on function-driven analysis (e.g. color changes, fluorescence, presence of inhibition zones) or on sequence-driven analysis (e.g. gene detection by PCR, hybridization with specific probes). For both approaches, high- or medium-throughput screening has to be considered when a high number of biological materials or targets are the prospection focus (Sette et al., 2013).

Endophytic fungi have been reported as interesting ASNase producers. Theantana et al., (2007; 2009), who investigated the major fungi present in Thailand medicinal plants, found the main producers of this enzyme in the genera *Colletotrichum*, *Eupenicillium*, *Fusarium*, *Penicillium* and *Talaromyces*. Marine environment, as well, appears to be a prolific source of ASNase-producing fungi. Thirunavukkarasu et al., (2011) found that this enzyme is secreted by seaweeds endophytes of the genera *Alternaria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Paecilomyces*, *Phaeotrichoconis*, *Phoma* and *Pithomyces*. Sudha, (2009) reported that 17 fungal strains recovered from mangrove soil were able to produce ASNase, and Sundaramoorthi et al., (2012) selected five fungal strains isolated from different soils from Arabian Sea that exhibited the same activity.

A list of yeasts and filamentous fungi able to produce ASNase in SF is provided in Table 3 (Alhussaini, 2013; Gupta et al., 2009; Gurunathan & Sahadevan, 2012; Kil et al., 1995; Kumar & Manonmani, 2013; Lapmak et al., 2010; Raha et al., 1990; Ramakrishnan & Joseph, 1996; Sarquis et al., 2004; Shrivastava et al., 2012; Tippani & Sivadevuni, 2012; Zia et al., 2013).

Tippani & Sivadevuni (2012) detected ASNase activities of 404.0 and 376.0 IU/mL for *Fusarium semitectum* and *Fusarium moniliforme* using proline and glucose as nitrogen and carbon sources, respectively, while maximum production by *Fusarium oxysporum* (360.0 IU/mL) occurred with sodium nitrate. These results appear to be a clear proof of the leading role of nutritional factors in SF by fungi.

As far as the members of the *Aspergillus* genus are concerned, *Aspergillus niger* (Mishra, 2006), *Aspergillus terreus* and *Aspergillus tamari* (Sarquis et al., 2004) were shown to have great potential to produce ASNase. Limiting the comparison only to the best results collected in the literature, *A. terreus* MTCC 1782 exhibited ASNase activity in the range 16.05–43.99 IU/mL when cultivated for 58–96 h in SF on Czapek–Dox medium (pH 6.0–6.3) supplemented with Asn, L-proline, glucose or sucrose at 30–35 °C and 140–160 rpm (Gurunathan & Sahadevan, 2012; Gurunathan & Renganathan, 2011). Lower ASNase activity was reported for *A. niger* (5.45 IU/mL) in the same medium (pH 6.5) supplemented with glucose and Asn at 35 °C and 120 rpm after 96 h (Zia et al., 2013) as well as for *A. flavus* (1.76 IU/mL) in a glucose-Asn medium (pH 4.5) supplemented with starch under static conditions at 30 °C (Patro et al., 2014).

As regards to other fungal genera, *Bipolaris* BR438 isolated from brown rice was reported by Lapmak et al., (2010) to be the best ASNase producer among other genera, exhibiting ASNase activity of 6.30 IU/mL when cultivated in the Czapek–Dox medium containing 1% Asn and 0.4% glucose at 30 °C for 72 h.

Regarding SSF by fungi, several studies attempted to maximize ASNase production using different substrates and conditions. These results are summarized in Table 4 (Hosamani & Kaliwal, 2011; Mishra, 2006; Nair et al., 2013; Rani et al., 2012; Uppuluri & Reddy, 2009; Uppuluri et al., 2013). The best enzyme producers in SSF belong to the *Aspergillus* genus. Rani et al., (2012) obtained maximum specific activity of 70.67 U/g with *Aspergillus* sp. KUFS20 using orange peel as a substrate, while *A. terreus* MTCC 1782 yielded no less than 253.0, 110.0 and 85.00 U/g on moistened pomegranate, wheat bran and coconut oil cake, respectively, after 72–120 h (Nair et al., 2013). Mishra (2006) reported, under aerobic conditions, a maximum ASNase specific activity of 40.90 U/g using *Glycine max* bran with 70% moisture content and a mean particle size of 1205–1405 µm, after 96 h of SSF by *A. niger* at pH 6.5 and 30 °C.

The highest ASNase specific activity was reported for *A. niger* (344.0 U/g) cultivated in SSF on agro-wastes in a column bioreactor using sesame (black) oil cake as a substrate at 32 °C, 0.4 vvm aeration and 22 cm bed thickness (Uppuluri & Reddy, 2009; Uppuluri et al., 2013).

These results, taken together with those of SSF by bacteria, are very promising from the ASNase activity viewpoint; however, since SSF requires complex media, it appears to be still very far from satisfying the high purity requirements of ASNase for pharmaceutical and medical applications. Furthermore, in our opinion, the large availability of molecular biology tools makes the screening of fungal isoforms, rather than the cultivation of filamentous fungi,

Table 4. Summary of fermentation conditions and results of L-asparaginase production by filamentous fungi in SSF.

Microorganism	Substrates/conditions	L-asparaginase production	Reference
A. niger	Bran of *Glycine max*; moisture content 70%; pH 6.5; 30 °C; 96 h	40.90 U/gds	(Mishra 2006)
	Column bioreactor; sesame (black) oil cake; aeration of 0.4 vvm; bed thickness of 22 cm; 32 °C	344.0 U/gds	(Uppuluri & Reddy 2009, Uppuluri et al. 2013)
Fusarium equiseti	Soya bean meal; particle size 3 mm; 0.5% glucose; 0.5% $(NH_4)_2SO_4$; 0.5% yeast extract; moisture 70%; 20% (v/v) inoculum; 45 °C; 48 h	8.51 IU/mL of crude extract	(Hosamani & Kaliwal 2011)
Aspergillus sp. KUFS20	Orange peel; 0.01 M phosphate buffer; moisture 50%; inoculum 10^6 spores/mL; pH 6.2; 30 °C; 96 h	70.67 U/gds	(Rani et al. 2012)
A. terreus MTCC 1782	Moistened pomegranate; inoculum 10^7 spores/mL; 30 °C; 120 h	253.0 U/gds	(Nair et al. 2013)
	Moistened wheat bran; inoculum 10^7 spores/mL; 30 °C; 72 h	110.0 U/gds	
	Moistened coconut oil cake; inoculum 10^7 spores/mL; 30 °C; 120 h	85.00 U/gds	

Units are different because studies report values expressed in different units. IU, international unit; gds, grams of dried substrate.

an interesting issue for future construction of a recombinant organism for ASNase production.

Downstream processing

Processes for producing therapeutic proteins have achieved substantial advances in the last decades. Nonetheless, purification processes deserve the same attention (Dutra-Molino et al., 2014). Downstream steps might reach 50–80% of the total production costs of proteins, and the best step combination can result in significant enhancement in terms of purification and process economy.

Protein precipitation

Separation by precipitation from an aqueous extract is the most traditional method to recover and purify biomolecules (Golunski et al., 2011). This method results in a temporary disruption of protein secondary and tertiary structures, leading to its precipitation. It is a technique easy to scale-up, with simple equipment requirements, low costs and possibility to use a large number of precipitants. Moreover, the precipitant agent can be recycled in the final process by distillation, evaporation or heat drying, thus reducing the environmental impact associated to its disposal.

ASNase recovery from different sources often employs precipitation. Several authors have purified ASNase up to apparent homogeneity by $(NH_4)_2SO_4$ precipitation (Amena et al., 2010; Basha et al., 2009; Gervais et al., 2013; Manna et al., 1995; Mishra, 2006; Moorthy et al., 2010). In general, partial purification starts with the addition of finely powdered $(NH_4)_2SO_4$ to the crude extract containing the enzyme up to saturation, causing ASNase precipitation. Salt concentration ranges from 35% to 100%, resulting in different yields depending on the ASNase source. Moorthy et al., (2010) recovered up to 96.2% ASNase from *Bacillus* sp. grown on glucose, with purification fold of 10.9 after precipitation. Salt precipitation is usually followed by centrifugation and column filtration to increase the purification yield. Production and purification of *Streptomyces gulbargensis* ASNase yielded 50.6%, with 1.8 purification fold. Purification was increased to 26.9-fold after precipitate was collected by centrifugation and dialyzed against buffer followed by Sepharcryl S-200 column filtration (Amena et al., 2010). Similar strategy was employed by El-Bessoumy et al., (2004) in the attempt to produce, isolate and purify ASNase from *P. aeruginosa* after SSF. Purification after $(NH_4)_2SO_4$ precipitation resulted in 5.2-fold purification and increased over five times after gel filtration through Sephadex G-100.

Other precipitant agents such as ethanol can also be successfully employed to pre-purify and concentrate the target biomolecule. Ethanol is widely produced in Brazil and worldwide (Golunski et al., 2011) and can be recycled after precipitation reducing the environmental impact.

Precipitation is one of the first steps in the downstream process and it is usually combined with traditional techniques to enhance biomolecules purification fold and process yield. Precipitation can also concentrate the target molecule reducing the volume for later stages (Glatz, 1990). Nonetheless, innovative techniques should be investigated aiming at faster and less expensive purification processes.

Liquid–liquid extraction

One interesting alternative to be exploited in separation science for biomolecules extraction/purification is liquid–liquid extraction (LLE) by aqueous two-phase systems (ATPS). LLE is defined as the removal of a solute from a liquid (or liquid mixture) phase when in contact with another immiscible or partially soluble liquid (or liquid mixture) where the component (solute) is preferentially soluble. Its partition can be improved using simple tools such as, for instance, the addition of affinity ligands (Albertsson, 1986; Lam et al., 2004). ATPS can be obtained when mixtures of water-soluble polymers are combined with another polymer or with certain inorganic salts above critical concentrations. They can also be formed using other materials such as surfactants, block copolymers and ionic liquids. Several physicochemical properties influence protein partitioning in

two-phase systems, namely isoelectric point, surface hydrophobicity and molar mass of system components. Also, partitioning depends on polymer or surfactant concentration, pH and salt addition (Albertsson, 1986).

Few attempts were made to employ ATPS to purify ASNase. Qin and Zhao (2003) described a combined strategy to release and separate ASNase from *E. coli* ATCC 11303 cells by aqueous two-phase micellar systems (ATPMS). Cells treated with 9.4% (w/v) K_2HPO_4 and 15% (w/v) Triton X-100 at 25 °C for 15–20 h released nearly 80% of the enzyme, while keeping whole. Considering the structure of *E. coli* cells and that ASNase is located in the periplasmic space, those authors proposed that Triton X-110 micelles might disrupt the outer membrane and cause the release of the enzyme from the periplasmic space. For this system (surfactant/phosphate/water), phase separation into a micelle-rich top phase and a salt-rich bottom phase was observed above critical micelle concentration. Most of the released enzyme was recovered in the bottom, phosphate-rich phase. In spite of the potential offered by ATPMS to release/purify ASNase, no data on purification factor or contaminants/whole cell partitioning behavior was reported.

Jian-Hang et al. (2007) proposed another strategy combining cell disruption by high-pressure homogenization and product capture by ATPMS for the extraction of intracellular ASNase from *E. coli*. They employed triblock copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO), PEO–PPO–PEO, to form primary ATPS for enzyme purification. ASNase purification via this novel *in situ* process resulted in increased enzyme yield (from 52% to 73%) and specific activity (from 78.60 to 94.80 U/mg) compared with the conventional process, including cell disruption, centrifugal clarification and subsequent ATPMS.

ATPS still needs further investigation to be employed for commercial ASNase purification, but the results obtained with this technique already point to its potential. Furthermore, we believe that there is a need to further investigate ATPS composed of ionic liquids for ASNase purification, which seems to be a particularly promising alternative (Souza et al., 2015).

Chromatography

Intravenous enzymes preparations such as ASNase require high levels of purity, thus a sequence of purification processes is necessary. A high degree of enzyme purity is also important from the viewpoint of process control, but steps required for purification, in general, result in enzyme activity loss and increased final cost (Gräslund et al., 2008).

ASNase from *P. aeruginosa* 50071 obtained by SSF was purified by $(NH_4)_2SO_4$ fractionation, Sephadex G-100 gel filtration and CM-Sephadex C50 ionic exchange, and the specific activity increased from 17.90 (crude extract) to 1900 IU/mg (final preparation) (El-Bessoumy et al., 2004). A similar method was used by Singh et al. (2013) to purify extracellular ASNase from the protease-deficient *Bacillus aryabhattai* ITBHU02 strain. After $(NH_4)_2SO_4$ fractionation and DEAE-Sepharose fast flow and Seralose CL-6B column chromatographic steps, the enzyme was 68.9-fold purified with specific activity of 680.47 U/mg. Warangkar and Khobragade (2010), who extracted and purified ASNase from *E. carotovora* by $(NH_4)_2SO_4$ fractionation (60–70%), followed by chromatographic steps using Sephadex G-100, CM cellulose and DEAE Sephadex, observed an increase in the enzyme specific activity from 1.36 to 1034 IU/mg, with a minimum mass loss and a final recovery of 36.5%.

Penicillium digitatum was also used to produce extracellular ASNase. After protein precipitation and desalting, gel filtration chromatography with Sephadex G-25, followed by Sephadex G-100, resulted in a 60.9-fold purification. Loureiro et al. (2012) purified ASNase from *Aspergillus* sp. by ion exchange chromatography (DEAE Sepharose) followed by gel filtration (Sephacryl S-200HR) at different flows, resulting in a 12% final yield and a 7.72 purification factor. An additional purification step by reapplying the pooled fraction to the same Sephacryl S-200HR column resulted in even lower yield (7.28%) with a purification factor of 10.7.

In recent work, ASNase has been obtained from the genus *Cladosporium*. Precipitation followed by DEAE cellulose ion exchange and subsequent size exclusion chromatography ensured a final specific activity of 83.3 U/mg (Kumar & Manonmani, 2013).

This overview reveals that protocols for ASNase purification from different sources lead to different purification performances; therefore, any comparison is not straightforward. Nonetheless, one can see that gel filtration and ion-exchange chromatography are the most employed purification steps. They are often preceded by precipitation with $(NH_4)_2SO_4$ as a first pre-chromatographic step, which, in our opinion, may be replaced by some ATPS.

ASNase formulation

ASNase PEGylation

Conjugation of proteins with polyethylene glycol (PEG), or PEGylation, has become a well-established technology in the field of biopharmaceutical formulations to increase half-life. It reduces the urinary excretion of a biomolecule (Yang et al., 2004) as well as its enzymatic degradation due to the increased steric bulk (Veronese & Pasut, 2005). In addition, PEGylated bioproducts often exhibit reduced affinity for the target receptor compared with the native precursor, which can lead to a lower clearance by target-mediated clearance mechanisms. Finally, the addition of PEG moieties might enhance the immunological profile of a biomolecule by reducing its ability to raise antibodies (Mehvar, 2000).

Since PEG polymers are highly hydrated, with 2–3 water molecules per ethylene glycol unit, their hydrodynamic radius is approximately 5- to 10-fold greater than would be predicted based on their nominal molecular weight (Harris & Chess, 2003), underlying a dramatic increase in the effective molecular size of PEG–protein conjugates. PEG provides protein protection from proteases and peptidases, by impairing access of proteolytic enzymes. Conjugation with PEG creates a hydration shell surrounding proteins, whose size determines the degree of hydration and depends on PEG structure, in that branched configuration has higher hydration level compared with the random coil one (Tirosh et al., 1998). Such a dynamic hydrated PEG shell is also responsible for protection from proteolysis. Despite this, the high flexibility

of the PEG backbone chain usually enables high affinity interactions between enzyme and substrate. Consequently, PEGylated therapeutic proteins may retain efficacy while acquiring greater stability in plasma (Fishburn, 2008).

PEG-ASNase was approved by the US Food and Drug Administration in 1994 for the treatment of patients exhibiting hypersensitivity to the native *E. coli* enzyme and in 2006 as a first-line treatment for patients with ALL. *E. coli* ASNase, one of the first PEGylated proteins, was produced by a random process coupling several 5 kDa PEG chains with the enzyme surface. As a result, polydispersity is considerable in PEG-ASNase formulations (Pasut et al., 2008).

Monomethoxylated PEG is generally used in protein conjugation because its monofunctionality yields cleaner chemistry. However, PEG diol impurities, with molecular weight approximately twice that of the monofunctional methoxy-PEG, are always formed in a percentage (1–10%) that rises with PEG molecular weight, hence increasing the polydispersity of conjugates (Veronese, 2001).

Enzyme PEGylation may be accompanied by loss of biological activity of the conjugate compared with the native enzyme, owing to sterical hindrance of the active site, which may be somehow prevented by site-specific modifications (Veronese & Pasut, 2005). In spite of these drawbacks, PEG-ASNase formulations have longer half-life compared with native enzymes (5 and 10 times longer than free *E. coli* and *Erwinia* enzymes, respectively) (Dinndorf et al. 2007) and increased thermostability (Soares et al., 2002). PEG-ASNase activity in plasma is detectable 1 h after injection and elimination half-life is 5.5–7.0 days, while native *E. coli* and *Erwinia* ASNases have elimination half-lives of 26–30 and 16 h, respectively (Avramis & Panosyan, 2005).

After intramuscular injection, PEG-ASNase level gradually increases, whereas that of Asn gradually falls over several days. Intravenous injection of PEG-ASNase is better tolerated and may lessen discomfort to patients and provide immediate Asn depletion. Although ASNase dosage strongly depends on its half-life, the effect of route of administration is not so evident (Avramis & Panosyan, 2005).

In spite of increased half-life, decreased dosage and frequency of PEG-ASNase, the degree to which PEGylation reduces the enzyme immune response is unclear, and there are reports where patients developed hypersensitivity to the PEGylated form. Antibodies against PEG were found in some patients with undetectable ASNase activity after receiving PEG-ASNase and were proposed as the cause of rapid clearance of conjugates and absence of enzyme activity in serum (Pasut & Veronese, 2009).

Associated with the longer half-life of PEG-ASNase in serum, and as a result of enzyme action, there is the risk of an increase of ammonia blood concentrations to clinically significant levels. Hyperammonemia, after ASNase therapy, was first reported by Leonard and Kay in 1986 (Heitink-Pollé et al., 2013), but the ASNases preparations they used (EcA and ErA) had short half-life, and ammonia was rapidly removed from circulation. Due to the longer PEG-ASNase half-life, the ammonia concentration could not return to normal levels between doses, and ammonia toxicity accumulated, with the appearance of hyperammonemia symptoms that disappeared after cessation of enzyme administration (Heitink-Pollé et al., 2013). Based on this background, despite the benefits of conjugating therapeutic proteins with PEG, in our opinion, drug pharmacodynamics and pharmacokinetics have to be better characterized to ensure therapeutics efficiency of PEG-ASNases with minimal negative side effects.

Freeze-dried formulations

Parenteral enzyme formulations must comply with their specifications during long-term storage. Water-dispersed enzymes, however, might be degraded through peptide bond hydrolysis and deamination, among other reactions, which are favored by a temperature increase (Singh et al., 2009). ASNase is susceptible to these reactions, which reduce its long-term or even short-term stability. For example, it was shown that partial deamination of ErA and EcA changes their isoelectric point and plasma half-life, although with no changes in enzymatic activity (Gervais et al., 2013).

Freeze-drying can prevent most water-related reactions by sublimating water from the frozen product under vacuum, also allowing sterile drying without heating or chemical sterilization. This process, however, suffers from other undesired effects such as cold denaturation, freeze denaturation and osmotic pressure increase due to dehydration and cryoconcentration (Singh et al., 2009), whose relative importance can be evaluated by splitting the process into freezing and drying.

As regards the freezing process, ErA activity was lost up to 40% when the enzyme was subject to freeze–thaw cycling, restored 24 h after freeze–thaw at −20 °C and partially restored when the freezing temperature reached −40 °C. Transient activity loss depends on the tetramer dissociation rate (Gervais et al., 2013), whereas cleavage of dissociated monomers leads to aggregation and permanent activity loss (Jameel et al., 1997). Reconstituted EcA (Elspar®) was shown to retain *in vitro* activity after cold storage for at least 14 days (4 °C) and freezing (−20 °C) for at least 6 months, besides resisting repeated freeze–thaw cycles (Wypij & Pondenis, 2013). Moreover, the presence of mannitol in Elspar® formulation may contribute to prevent cold denaturation.

Freeze-drying without protectants is rarely an option for proteins. Hellman et al. (1983) tested several protectants at different concentrations, which had been submitted to the same freeze-drying procedure as ErA. Glucose, mannose and sorbitol showed 100% tetramer and activity retention after reconstitution at pH 10.0, while 20% and 50% retentions were observed in the absence of any protectant and in the presence of mannose contained in commercial formulations, respectively. Protein retention reached a maximum value when the enzyme was freeze dried without protectants and reconstituted at pH 7.5. Conversely, Izutsu et al., (1994) observed 50% activity retention with the naked enzyme and proposed mannitol as a good protectant under natural conditions, as long as the amorphous form is maintained. Such discordance might be related to different freeze-drying protocols employed by these research-groups, in that the former froze the product at −35 °C for 24 h, while the latter quickly froze with liquid nitrogen.

The collapse temperature is an essential parameter for freeze-drying; formulations must in fact be dried below their

collapse temperature to maintain stability, easy reconstitution and dry cake form. Adams & Ramsay, (1996) characterized ErA formulations with a variety of protectants in terms of collapse temperature, cake structure and activity/tetramer retention. Lactose was shown to be the most suitable protectant among those tested, together with lactose/mannitol combination. These authors performed a cycle optimization to reduce the drying time, based on the collapse temperature. The effectiveness of lactose as a protectant during ErA freeze-drying confirmed the results of a previous study that demonstrated the potential of other disaccharides as enzyme stabilizers such as trehalose and maltose (Adams & Irons, 1993).

Lyoprotection mechanisms are not fully elucidated. Based on the current hypothesis that protectants substitute water in protein hydration shell, Ward et al. (1999) proposed that each highly polar residue of ASNase requires one molecule of protectant, and this way they were able to estimate sugar concentration, but failed to predict PEG amount.

Instead of adding protectants, it is possible to conjugate them to improve thermostability, among other properties. For instance, PEGylated EcA activity was entirely recovered when reconstituted after freeze-drying (Soares et al., 2002). EcA conjugation with inulin, on the other hand, led to improvement in freeze-thawing resistance with no significant change in freeze-drying (Tabandeh & Aminlari, 2009).

Literature on EcA freeze-drying is scarce and outdated, while ErA is more explored. Papers are generally broken down into formulation screening or process parameters, with large disparities in freeze-drying methodology. Patents were deposited but, for obvious reasons, information is not straightforward. So far, no model is adequate enough to predict freeze-drying behavior of protein solutions; therefore, empirical testing is still needed to obtain an adequate formulation.

Structural features, undesirable characteristics and protein engineering of bacterial ASNases

Despite being a widely used drug, ASNase possesses a secondary L-glutaminase activity (GLNase), and several side effects are associated with ASNase-based treatments, including immunological reactions, hepatotoxicity, neurotoxicity, coagulation abnormalities, among others. Additionally, its administration to patients often results in a rapid decay of circulating ASNase levels, leading to high administration frequency. Determination of crystallographic structure of several ASNases has assisted decisively in a better elucidation of the enzyme features and the catalytic process. Moreover, these data have currently enabled approaches involving rational enzyme engineering based on structural data and *in silico* methods, aiming to obtain more efficient and specific bacterial ASNases.

Enzyme structure and catalytic mechanism

EcA was the first bacterial ASNase whose high-resolution crystallographic structure was determined (Swain et al., 1993). Structural studies revealed a well-organized homotetrameric enzyme, with each monomer containing ~330 amino acids arranged in two domains (N- and C-terminal), both belonging to the α/β class. The N-terminal domain comprises the residues 1–190 and is connected to the smaller C-terminal domain (213–326) by a large linker (191–212) (Figure 1A). Monomers are able to associate tightly with each other forming intimate dimers characterized by an extensive interface between the subunits that are held together by several interactions, mainly van der Waals and electrostatic interactions (Figure 1B). Finally, the association of two dimers results in the tetrameric biological unit, which is kept together by molecular interactions similar to those found in homodimers (Figure 1C) (Jaskólski et al., 2001; Swain et al., 1993). Several macromolecular ASNase structures are available at Protein Data Bank (PDB) (http://www.rcsb.org), including ErA (Lubkowski et al., 2003; Sanches et al., 2003), which shares high structural homology with the *E. coli* counterpart (r.m.s.d. = 0.89) (Figure 1D).

ASNases active sites are located at the interface of intimate dimers, with each intimate dimer containing two active site pockets formed by amino acids from both subunits (Palm et al., 1996; Swain et al., 1993). Structural and functional studies revealed that the so-called catalytic triad composed of three polar amino acids, namely Thr-Lys-Asp (Thr^{89}, Lys^{162} and Asp^{90} in EcA), is essential for enzyme activity (Jaskólski et al., 2001).

Examination of ASNase structure with ligand molecules in the active site revealed the formation of an intricate hydrogen network with ligands (Figure 2A and B) and disclosed two additional residues of importance for the catalytic mechanism (Thr^{12} and Tyr^{25} in EcA) (Michalska & Jaskolski, 2006). These residues are located in a large loop (amino acids 10–32 in EcA) that operates as a lid for the active site, probably assisting the correct substrate binding and thus favoring catalysis (Figure 2C and D).

Immunological side effects and enzyme instability

ASNase administration can promote a number of harmful side effects including immunological responses, ranging from allergic reactions to fatal anaphylactic shock, coagulation disorders, pancreatitis, hyperglycemia, hepatotoxicity and protein synthesis inhibition (Rizzari et al., 2013). As far as the immunological side effects are concerned, the decay of ASNase antitumoral activity is directly associated with the production of ASNase antibodies by the patient, which leads to the drug clearance from the bloodstream and reduces the treatment efficacy. In this respect, early studies have shown high circulating levels of ASNase by ELISA with low enzyme activity, which was initially attributed to ASNase denaturation (Asselin et al., 1993). However, more recent studies suggest that ASNase clearance may be a result of protease cleavage (Offman et al., 2011). Protein stability and immunogenic effects are apparently closely related. The proteolytic cleavage of ASNase may be responsible for additional epitopes exposure, which are involved in the patients' immune response (Offman et al., 2011; Patel et al., 2009). In this respect, cysteine proteases such as asparagine endopeptidase (AEP) and cathepsin B are able to hydrolyze bacterial ASNase.

ASNase antibody production has been observed in 60% of patients treated with EcA. ASNase hypersensitivity clinical symptoms include anaphylaxis, pains, angioedema, hives, rash and pruritus. Hypersensitivity occurs more frequently

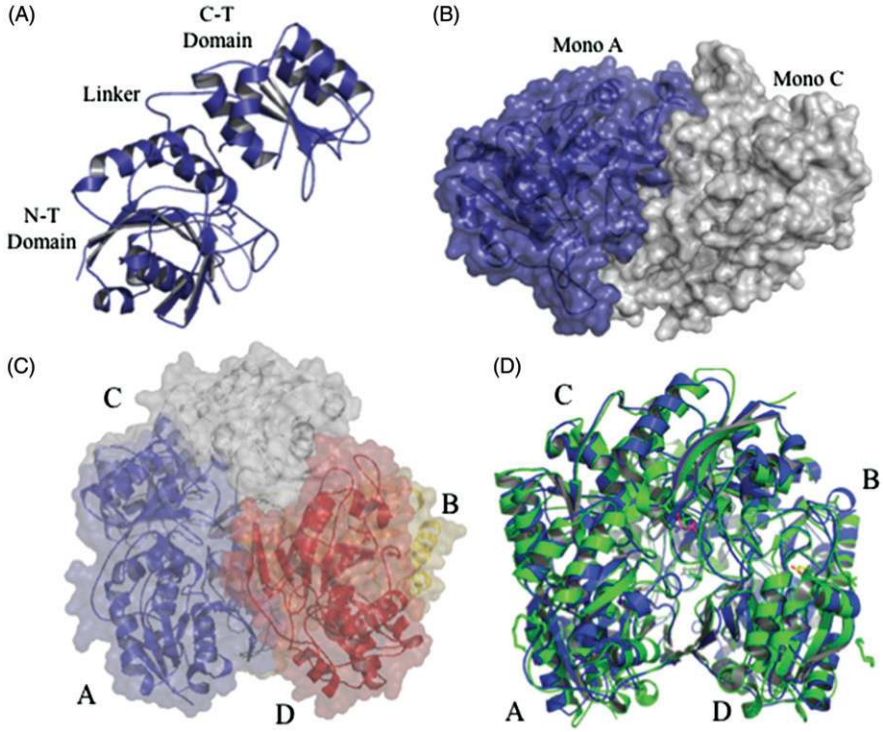

Figure 1. Structural features of bacterial ASNase. A) Cartoon representation of the ASNase monomer from *E. coli*, composed by the N-terminal and C-terminal domains connected by a large loop. B) Molecular surface of intimate dimer of EcA enzyme with the monomers in different colors. C) Homotetrameric quaternary structure of EcA. Inside the molecular surface is depicted the cartoon representation of the enzyme. D) Superposition of ASNases crystallographic structures from E. coli and *E. chrysanthemi*. Molecular graphics were generated by PyMOL software (http://www.pymol.org) and the coordinates 3ECA (*E. coli*) and 107J (*E. chrysanthemi*) from the PDB (http://www.rcsb.org).

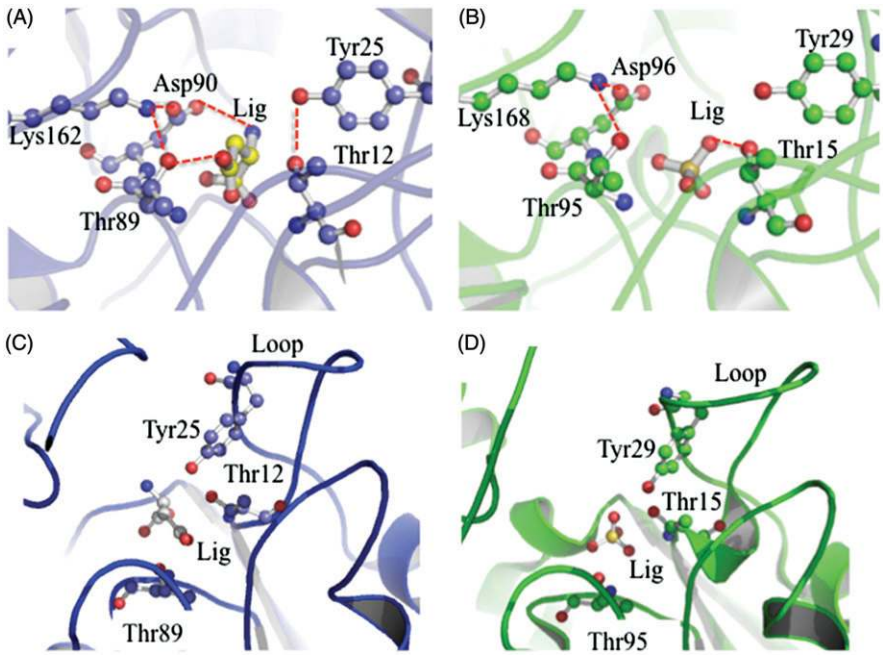

Figure 2. ASNases active site pocket structural features and ligand binding. The active site region of EcA (A) and ErA enzyme (B) reveals extensive structural similarities of the amino acids involved in catalysis (Thr^{12}, Tyr^{25}, Thr^{89}, Asp^{90} and Lys^{162}, in *E. coli*, and Thr^{15}, Tyr^{29}, Thr^{95}, Asp^{96} and Lys^{168} in *E. chrysanthemi*). Detail of the active site region of EcA (C) and ErA (D) showing the position of the loop at the entrance of the active site pocket. The representations are in cartoon and the catalytic amino acids are represented by balls and sticks. Molecular graphics were generated by PyMOL software (http://pymol.org) using the coordinates 3ECA (*E. coli*) and 107J (*E. chrysanthemi*) from the PDB (http://rcsb.org).

when the treatment is interrupted or discontinued, with children presenting less hypersensitivity and antibody production when compared to adolescents and adults (Pieters et al., 2011; Shrivastava et al., 2015).

Replacement or modification of ASNases to mitigate the immune side effects

The immunological side effects of ASNases can be partially minimized using enzymes from different bacteria (e.g. replacing EcA for ErA) (Avramis & Tiwari, 2006). In addition, new experimental protocols of enzyme encapsulation into erythrocytes or entrapment into liposomes showed lower side effects (Agrawal et al., 2013; Kwon et al., 2009; Patel et al., 2009).

As mentioned before, three ASNase formulations are used to treat ALL and other lymphoid malignancies, namely EcA, ErA and PEGylated EcA. Native EcA is the most commonly used worldwide and considered first-line therapy in Europe, with usual dosage of 6000 IU/m^2 thrice a week and

elimination half-life of 26–30 h. PEGylated EcA, which is the first-line therapy in USA, has a half-life (5.5–7.0 days) five times longer than native EcA and, consequently, its dosage is lower (2000–2500 IU/m^2 every 2 or 4 weeks). ErA has been used at a dosage of 6000 IU/m^2 thrice a week as a therapeutic option in patients with side effects from EcA, because it elicits lower immunological response and induces minor coagulation disorders. The main disadvantage of ErA is its half-life of only 16 h (Shrivastava et al., 2015).

Side effects and GLNase activity

An important side effect of ASNase treatment is related to the nonspecific ASNase amidohydrolase activity. Patients treated with bacterial enzymes also exhibit low levels of L-glutamine (Gln) in the bloodstream, resulting in decreased levels of protein synthesis (Offman et al., 2011). Despite the significantly lower k_m of ASNase amidohydrolase activity for Asn (30-fold lower in EcA) (Avramis & Tiwari, 2006), Gln represents 50% of all free amino acids in the body being an important nitrogen source (Ramya et al., 2012). The general effect of lowering protein synthesis is directly associated with several side effects, among which are immunosuppression, thromboembolism and neurological disorders (Kafkewitz & Bendich, 1983; Villa et al., 1986).

In spite of toxicity, Offman et al. (2011) demonstrated, using molecular dynamics and site direct mutagenesis, that GLNase activity of EcA is necessary for antitumor effect, and similar results were observed for *Helicobacter pylori* ASNase (HpASNase) (Parmentier et al., 2015). However, GLNase activity of EcA was shown to be irrelevant for antitumor effect against cell lines that do not express asparagine synthetase (ASNS), but required in cell lines with high ASNS levels (Chan et al., 2014).

Resistance to ASNase treatment

Many patients are refractory to treatment with ASNases, which is ultimately related to differences in genetic background and gene expression profile, including especially NF-κβ related genes. This indicates very complex genetic, biochemical and cellular relationships involved in ASNase resistance mechanism (Chien et al., 2015; Holleman et al., 2004). Several studies demonstrated that ASNase resistance is associated to ASNS up-regulation in ALL cells that are refractory to bacterial ASNase treatment (Chan et al., 2014; Chien et al., 2015). Conversely, the decrease of *asns* mRNA expression levels enhances the sensitivity of HL-60 cells to EcA treatment (Hashimoto et al., 2009).

Studies with ASNS inhibitors also indicate the role of this enzyme in the resistance to ASNase treatment. For example, analogues of adenylated sulfoximine, an intermediate of ASNS catalytic mechanism, are able to enhance the *in vitro* cytotoxicity of bacterial ASNases to resistant cell lines (MOLT-4) (Gutierrez et al., 2006; Ikeuchi et al., 2009; Ikeuchi et al., 2012).

As discussed above, recently some authors have argued that glutaminase activity of ASNases is fundamental for cytotoxicity against several types of ALL cancer cells (Chan et al., 2014; Chien et al., 2015), so the Gln intracellular levels may be also important for treatment efficacy. In this context, among several drugs used in combination with ASNases are the glucocorticosteroids, stimulators of glutamine synthetase (GLNS) expression (Gaynon & Carrel, 1999). Despite the lack of studies on the effects of GLNS in ALL cells, inhibition of Gln uptake was recently suggested to play a role in the treatment of acute myeloid leukemia, since bacterial ASNase up-regulates GLNS expression. Additionally, GLNS inhibition by methionine-L-sulfoximine in six different human sarcoma cell lines with distinct sensitivity to ASNase increased cytotoxicity (Tardito et al., 2007). Nonetheless, more studies on GLNase activity of ASNases are necessary to verify the molecular mechanisms involved in enzyme regulation and cancer cell proliferation.

Mutational studies and rational enzyme engineering

Several mutational approaches were performed in ASNase aiming to investigate aspects such as catalysis, substrate binding, turnover and affinity, antigenicity and oligomerization, among others. Table 5 summarizes the structural effects of amino acids mutations in EcA (Aung et al., 2000; Borek et al., 2014; Chan et al., 2014; Derst et al., 1992; Derst et al., 1994; Derst et al., 2000; Harms et al., 1991; Jaskólski et al., 2001; Jianhua et al., 2006; Mehta et al., 2014; Offman et al., 2011; Palm et al., 1996; Verma et al., 2014; Wehner et al., 1992).

Excluding the pioneering studies involving covalent modifications by inhibitors, peptide sequencing and other techniques to identify amino acids involved in catalysis (Harms et al., 1991; Wehner et al., 1992), the vast majority of the site mutagenesis approaches were in fact performed after the determination of EcA crystallographic structures. These include structures showing ligands at the active site pocket (Jaskólski et al., 2001), demonstrating the importance of protein structure knowledge to rationally evaluate the amino acids involved in substrate binding and catalysis as well to plan replacements strategies.

The amino acids substitutions that affect the kinetic parameters of bacterial ASNAses are concentrated, close to the active site, in the intimate dimer interface (Figure 3A and B). However, there is a lack of mutational studies on the dimer complementary region, which is also part of the active site pocket (Figure 3C and D). This observation is important, since the active site volume is closely related to substrate(s) binding, aspects that will be addressed later.

The molecular surface mapping of amino acids substitutions that cause alterations of enzyme stability reveals that several amino acids are located at the enzyme surface, some of them between the dimers (Asn^{24}, Asp^{138} and Tyr^{250}) or tetramer contacts (Asn^{37}, Asn^{124}, Lys^{139}, Tyr^{181} and Lys^{207}) (Figure 4). Rational efforts were also performed aiming to enhance enzyme stability and half-life. Replacement of Asn^{24} by Ala or Thr, located in the lid loop containing Tyr^{25}, increased enzyme stability, rendering higher resistance to AEP and increasing enzyme activity (Offman et al., 2011).

Regarding antigenicity, hydrophilic protein regions associated with long side chain-amino acids are closely related to antigenic sites. Studies involving EcA antigenicity showed that several amino acids fit into the characteristics described above (Tyr^{176}, Arg^{195}, Lys^{196}, His^{197} and Lys^{288}) (Figure 4A–

Table 5. Summary of the amino acid mutations and the functional/structural effects on *E. coli* L-asparaginase.

Amino acid	Substitution(s)	Functional and or structural effects	Reference
G11	V11 or L11	Catalysis almost abolished	(Derst et al. 2000)
T12	A12	ASNase activity reduced to 0.01%	(Harms et al. 1991)
T12	A12	ASNase activity reduced to 0.04%	(Derst et al. 1992)
T12	S12	Normal activity but altered substrate specificity	
N24	G24	Decrease of ASNase and GLNase activities	(Offman et al. 2011)
N24	A24 or T24	AEP resistant enzyme	
N24/R195	A24/S195	Decay of ASNase activity/slight decrease of GLNase activity	
N24/R195	T24/S195	Abolishment of ASNase activity/slight decrease of GLNase activity	
N24/Y250	A24/L250	Abolishment of ASNase activity/slight decrease of GLNase activity	
N24/Y250	T24/L250	Abolishment of ASNase activity/slight decrease of GLNase activity	
Y25	F25	High decrease of GLNase activity	(Jaskólski et al. 2001)
Y25	A, H or F	ASNase activity reduced to 0.1–0.2%	(Derst et al. 1994)
V27	L27 or M27	Reduction in K_m values/moderate reduction of k_{cat} for Asn	(Derst et al. 2000)
G57	A57	Little effect on substrate specificity/reduction of activity with AHA, Asn and Gln	
G57	V57 or L57	Strong reduction of k_{cat} for all substrates/low alteration in substrate specificity	
Q59	G59 or A59	Strong increase in K_m for AHA/Increase of binding constants for aspartate/Interference in GLNase activity to larger extent than the turnover of AHA or Asn.	
Q59	E59	Reduction in k_{cat} for AHA, Asn and Gln to about 10% of the wild-type/No differences between ASNase and GLNase activities	
Q59	L59	High decrease of GLNase activity/slight effect on ASNase	(Chan et al. 2014)
Q59	N59	Decrease (∼80%) of GLNase and ASNase activities	
Q59	H59	Decrease (∼50%) of GLNase and ASNase activities	
Q59	L, F, C, T, S, Y, W, V, I, A, M, P, G, R or K	High decrease of GLNase activity	
W66	Y66	More effective killing of ALL cells/Induction of apoptosis in lymphocytes derived from ALL patients/Rapid depletion of Asn/down-regulation of the transcription of asparagine synthase	(Mehta et al. 2014)
W66/Y25	Y66/W25	Decrease of k_{cat} for Asn/reduction of active site loop movement	(Aung et al. 2000)
W66/Y176	Y66/W176	Resistance to chemical denaturation/Increase of V_{max}/K_m for AHA	(Verma et al. 2014)
W66/Y181	Y66/W181	Higher susceptibility to chemical and thermal denaturation	
H87	A87, L87, K87	No substantial changes of K_m; Moderate decrease of k_{cat} for Asn	(Wehner et al. 1992)
G88	A88 or I88	Asn catalysis almost abolished	(Derst et al. 2000)
T89	V89	Aspartate aspartyl moiety was found to be covalently bound to Thr-12	(Palm et al. 1996)
D90	E90	Decrease of k_{cat} and increase of K_m for Asn	(Borek et al. 2014)
T119	A119	Decrease of Asn activity with no marked effect on substrate binding	(Derst et al. 1992)
S122	A122	No effect	
Y176	F176	More effective killing ALL cells/Induction of apoptosis in lymphocytes derived from ALL patients/rapid depletion of Asn and down-regulation of ASNS transcription/ decrease of GLNase activity/Increase of V_{max}/K_m for AHA	(Mehta et al. 2014, Verma et al. 2014)
Y176	S176	Decrease of GLNase activity/Increase of V_{max}/K_m for AHA	
Y181	S181 or C181	Instability of tetramer	(Derst et al. 1994)
H183	L193	Sensitivity to urea	(Wehner et al. 1992)
R195/K196/H197	A195/A196/A197	Reduction in antigenicity	(Jianhua et al. 2006)
H197	L197	No substantial changes of K_m; moderate decrease of k_{cat}	(Wehner et al. 1992)
N248	D248 or E248	Reduction of k_{cat} for Asn	(Derst et al. 2000)
N248	A248	Loss of transition state stabilization of Gln hydrolysis twice as high as that for Asn and more than three times higher than for AHA	
N248	G, D, Q or E	Moderate reduction of maximum velocity of Asn hydrolysis/Strong impairment of Gln turnover.	
Y250	F250	No effect	(Derst et al. 1994)
K288	R288	Decrease of GLNase activity	(Mehta et al. 2014)

(*continued*)

Amino acid	Substitution(s)	Functional and or structural effects	Reference
K288/Y176	S288/F176	Reduction of antigenicity and immunogenicity/Rapid depletion of Asn and down-regulation of ASNS transcription/decrease of GLNase activity	
K288/Y176	R288/F176	Decrease of GLNase activity	
Y289	F289	No effect	(Derst et al. 1994)
Y326	Stop	Instability of tetramer	

AEP, asparagine endopeptidase; AHA, L-aspartic acid beta-hydroxamate; ALL, acute lymphoblastic leukemia; Asn, L-asparagine; ASNase, L-asparaginase; ASNS, L-asparagine synthase; Gln, L-glutamine; GLNase, L-glutaminase; Thr, Threonine.

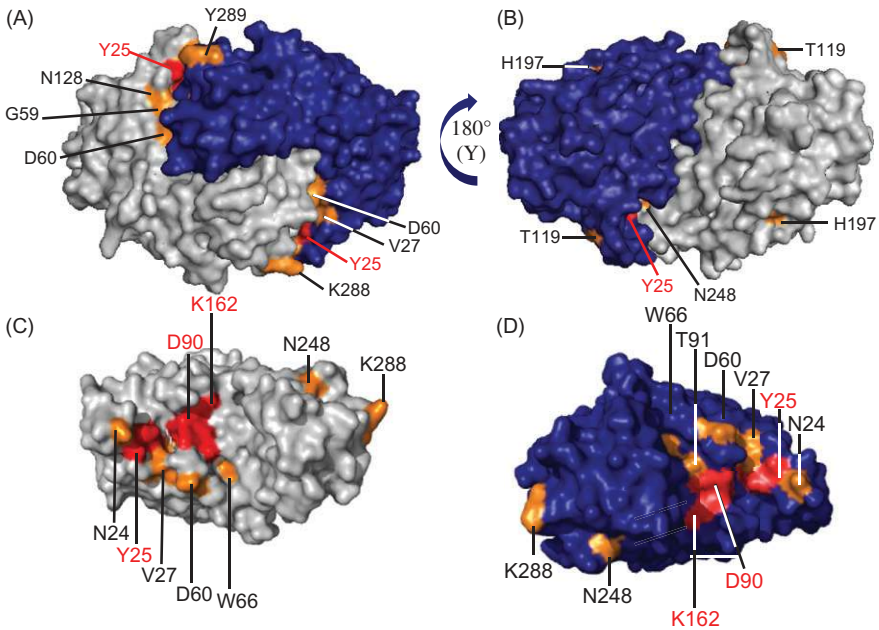

Figure 3. *E. coli* ASNase surface structural representations of mutational approaches affecting enzyme activity. In A) and B) are represented the EcA dimer with the monomers colorized in different colors, revealing the positions of amino acids which substitutions resulted in altered enzyme activity over the substrates (e.g. Asn, Gln and AHA). C) and D) EcA monomer representations showing the amino acids in the monomers interfaces. Amino acids involved in catalytic activity are also detached on the enzyme surface (Y^{25}, D^{90} and K^{162}). Molecular graphics were generated by PDB coordinates 3ECA (*E. coli*) (http://www.rcsb.org).

D) (Jianhua et al., 2006; Mehta et al., 2014). Additionally, approaches using two distinct techniques to identify immunogenic peptides report an EcA fragment containing several bulky amino acids as the most immunogenic one (Werner et al., 2005). Interestingly, analysis of EcA tetramer structure reveals that some amino acids involved in the antigenicity are buried in the enzyme structure (Figure 4E and F). It is likely that, at very low concentrations such as those used in therapy, in addition to the tetrameric form, there are significant amounts of dimers with additional accessible residues, as previously demonstrated *in vitro* (Werner et al., 2005).

Recently, Mehta et al. (2014) demonstrated that EcA double substitution of $Lys^{288}Ser/Tyr^{176}Phe$ decreases significantly the enzyme immunogenicity, since the patient antibodies binding was reduced to less than 40%. As expected, Lys^{288} is located at the tetramer surface (Figure 4E and F), but curiously Tyr^{76} (Figure 4A and B) is buried between the dimers of the tetramer. Again, the tetramer dissociation may be related with this phenomenon.

As described previously, several ASNase undesirable characteristics have been ascribed to its GLNase activity (Aghaiypour et al., 2001; Chan et al., 2014; Derst et al., 2000; Mehta et al., 2014). In ErA, substitution of Glu^{63} and Ser^{254} led to a decrease in GLNase activity (Derst et al., 2000). In EcA, substitution of Asp^{248} by Ala revealed an effective decrease in Gln hydrolysis (Derst et al., 2000). However, the mutant also showed a significant decrease in Asn hydrolysis (about ~12% of the wild type).

Another study using molecular dynamics simulations combined with structural analysis and site directed mutagenesis demonstrated that the double substitution $Asn^{24}Ala$ and $Tyr^{250}Leu$ resulted in negligible GLNase activity and ~30% decrease of ASNase activity (Offman et al., 2011). As mentioned before, Asn^{24} is located close to Tyr^{25} from the lid loop (Figure 5A), and its substitution by Ala or Thr is related with proteolytic resistance to AEP. The GLNase activity decrease was associated with the active site cavity volume ($Asn^{24}Ala$, substitution) and tetramer compactness ($Tyr^{250}Leu$, substitution) (Offman et al., 2011). It is noteworthy that the cytotoxicity of mutant EcA was significantly lower than that of the wild type enzyme. The authors argued that EcA dual activity is also associated with therapeutic toxicity, since mutants with lower GLNase activity also exhibited lower cytotoxicity (Offman et al., 2011).

Chan et al. (2014) also investigated additional factors of cytotoxicity of EcA mutants deficient in GLNase activity. Molecular dynamics simulation approaches combined with saturation site directed mutagenesis allowed the authors to

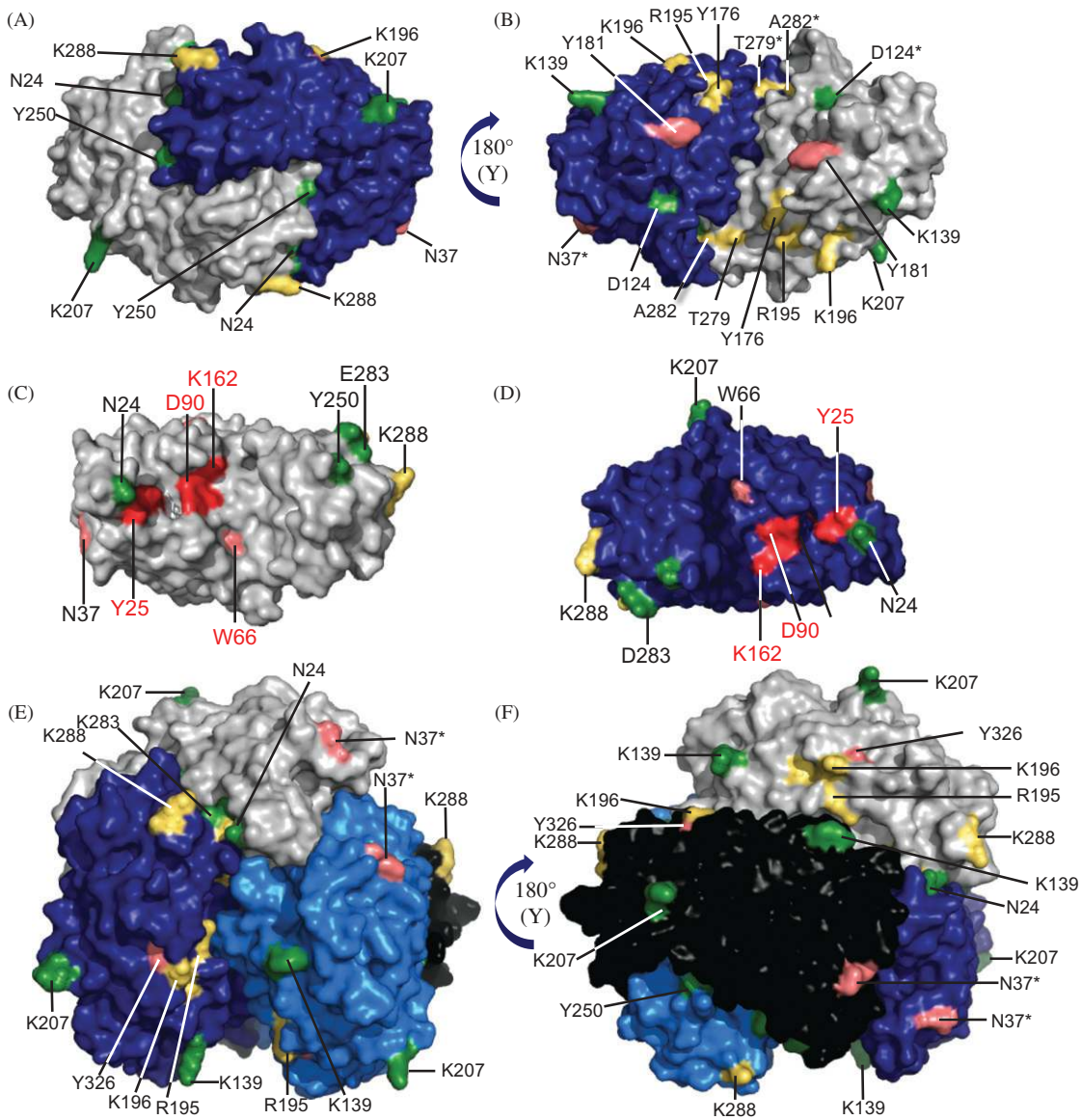

Figure 4. EcA structural surfaces representations of mutational approaches affecting enzyme stability and antigenicity. Surface mapping of amino acids whose replacement increase (N^{24}, $D^{124}*$, K^{139}, K^{207}, Y^{250} and D^{283}) or decrease ($N^{37}*$, W^{66}, Y^{181} and Y^{326}) the thermal or proteolytic enzyme stability or antigenicity decrease (Y^{176}, R^{195}, K^{196}, $T^{279}*$, A^{282} and K^{288}). The amino acids related to the catalytic activity are also depicted in the figure (Y^{25}, D^{90} and K^{162}). The asterisk (*) denotes amino acids substitutions performed in other bacterial species. In A) and B) are represented the EcA intimate dimer. C) and D) EcA monomer representations showing the amino acids in the monomers interfaces. In E) and F) are represented the tetrameric enzyme in different orientations. Molecular graphics were generated by PDB coordinates 3ECA (*E. coli*).

identify an EcA glutamine residue (Gln^{59}) in the active site pocket able to perform saline interactions with the ligand (Figure 5B). It was demonstrated that the mutant enzyme carrying Gln^{59} substitution by a Leu (EcA^{Q59L}) retains ~60% of ASNase activity, but no detectable GLNase activity.

The mutant EcA^{Q59L} was able to kill efficiently cell lines with low ASNS levels, but did not display cytotoxicity against cell lines with high expression of this enzyme (Chan et al., 2014). ASNS is able to catalyze the conversion of aspartate and glutamine to asparagine and glutamate through an ATP-dependent reaction, thus supplying ALL cells with Asn. Some studies report that ASNase-resistant forms of the disease may be associated with ASNS up-regulation in some patients (Richards & Kilberg, 2006). Nevertheless, the demonstration that EcA variants without GLNase activity are able to kill cancer cells with low ASNS levels is very important and may represent a promising therapy with less side effects for patients affected by tumors with this characteristic (Chan et al., 2014).

Recently, Verma et al. (2014) demonstrated that substitution of EcA Tyr^{176}Phe and Trp^{66}Tyr residues promotes a substantial decrease of GLNase activity, but ASNase activity is almost the same as that of the wild type enzyme. Tyr^{176} and Trp^{66} are very far from the enzyme active sites, at both dimers, and the effects of their replacements were related to an intricate network of indirect molecular interactions, which affects the enzyme quaternary folding with consequences in its activity (Figure 4). In subsequent studies, it was demonstrated that, contrary to the wild type enzyme, the mutant enzymes EcA^{W66Y}, EcA^{Y176F} and $EcA^{K288S/Y176F}$ are able to down-regulate ASNS expression, however the reasons for this phenomenon are not yet understood (Mehta et al., 2014).

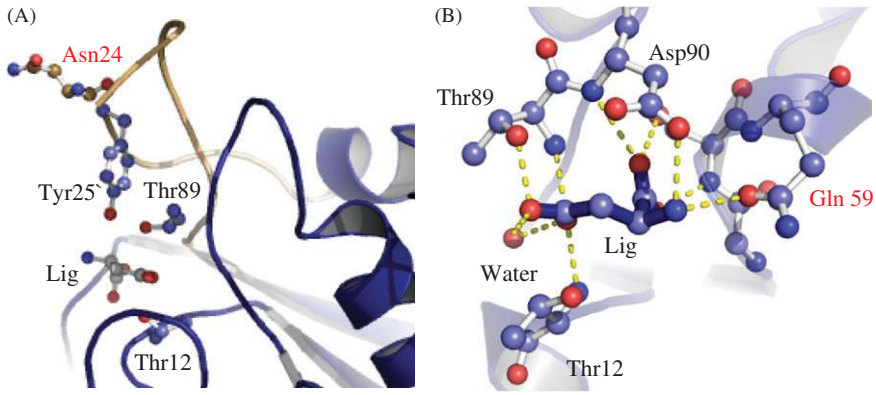

Figure 5. Amino acids involved in EcA enzyme stability and activity. A) Representation of the ASNase active site region of *E. coli* denoting the position of residues Thr^{12}, Asn^{24}, Tyr^{25}, Thr^{89} and the ligand (aspartate). The *E. coli* enzyme (PDB code 1NNS) is represented in cartoon. The active site amino acids and the ligand are represented by balls and sticks. B) Molecular binding of aspartic acid molecule in EcA, with polar interactions depicted as dashed lines. The Gln^{59} replacement results in a residual GLNase activity. Molecular graphics were generated by PyMOL (http://www.pymol.org).

The studies presented in this section provide amazing examples of how EcA rational protein engineering can assist in a better understanding of catalytic mechanisms, substrate binding, enzyme folding and their relationship with cytotoxicity and immunogenicity. Engineered enzymes or new variants derived from studies presented here may have high potential for future alternative therapeutic treatments using recombinant ASNAses. Additional studies involving structure determination and analysis of mutant enzymes with improved properties should provide a better understanding of EcA functional and structural features and open perspectives of creating new enzymes with personalized characteristics for different groups of patients. In addition to the rational modification based on structure analysis, emerging approaches based on computational analyses involving molecular dynamics, docking of substrates, among others *in silico* techniques, have proven to be very promising to guide rational enzyme modifications.

Concluding remarks and future perspectives

In general, ASNase is mainly produced by bacterial fermentation, and the native and pegylated forms of ASNase continue to be the most used in ALL therapy. Despite the benefits of conjugating ASNase with PEG, parallel negative side effects could be solved, in our opinion, through a better characterization of pharmacodynamics and pharmacokinetics, which would increase drug efficiency.

Nonetheless, due to ASNase importance in the treatment of several types of cancers, in particular leukemia, it is essential to search for new sources of this enzyme in order to increase its availability as a drug and reduce side effects. Considering the advantages of the use of microorganisms in bioprocesses and that very little is known regarding the magnitude of microbial diversity, they may be considered a target source of genomic innovation in the search for new ASNases with improved properties compared with those currently employed in therapy. In this sense, more effort should be devoted, in our opinion, to the screening of new ASNase-producing microorganisms.

Rational protein engineering based on protein structure is another promising strategy to produce ASNases with improved pharmacodynamics, pharmacokinetics and toxicological profiles. Indeed, approaches involving site directed mutagenesis of residues in the active site were able to produce recombinant enzymes with good ASNase activity, but negligible GLNase activity. Additional procedures involving the introduction of structural disulfides and deletion of proteases cleavage sites may allow the production of more robust enzymes. There is little information on *Saccharomyces cerevisiae* ASNase and, giving the ease of cultivation and possibility of genetic manipulation of this yeast, we believe that such an enzyme deserves to be better investigated as an alternative to the existing bacterial ASNases. In particular, special attention should be paid to its better structural and kinetic characterization as well as to the rational engineering of the yeast enzymes by means of site-directed and random mutations.

One interesting technological approach that may contribute to improve ASNase production by recombinant microorganisms is the metabolic flux analysis (MFA), a powerful tool to estimate the metabolic state constrained by exchange of nutrient fluxes between cells and environment (Antoniewicz, 2013). This analysis has been successfully used to identify key nodes in the primary metabolism, which are characterized by significant changes to the partitioning of the flux under different conditions, and thus it can be considered as a potential control point manipulation (Boghigian et al., 2010; Goudar et al., 2014). Additionally, *Pichia pastoris*, which has been developed as an excellent host for heterologous genes using alcohol oxidase as a promoter, has potential for high cell density cultivations with high levels of protein expression and efficient secretion (Cregg et al., 2000). Therefore, MFA applied to ASNase production by recombinant *P. pastoris* seems to us an interesting alternative to be investigated, with determination of optimal culture conditions in terms of temperature, methanol concentration and pH. This may be achieved through modeling of metabolic fluxes related to methanol-metabolizing pathway, which is important in the induction of the heterologous protein as well as yeast growth (Anasontzis et al., 2014).

The information gathered here also demonstrates a gap on novel alternatives and optimized protocols for ASNase bioproduction and purification. Therefore, research efforts should be addressed to these topics, with special focus on cultivation parameters and novel and cheaper purification strategies such as liquid–liquid extraction with ionic liquids. We hope that, in a near future, novel alternatives to bacterial ASNases can be available for ALL treatment, with better therapeutic results and less side effects.

Declaration of interest

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