Enzyme engineering strategies for the bioenhancement of L-asparaginase used as a biopharmaceutical

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Running Title

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Abstract

Over the past few years, there has been a surge in the industrial production of recombinant enzymes from microorganisms. This is due to their catalytic characteristics. L-asparaginase is an enzyme belonging to the class of amidohydrolases that catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. It has been widely investigated as a biologic for its antineoplastic properties in the treatment of acute lymphoblastic leukemia. The demand for it is primarily fulfilled through recombinant enzymes sourced from *Escherichia coli* and *Erwinia chrysanthemi*. However, the presence of immunogenic proteins in L-asparaginase sourced from prokaryotes has been known to result in adverse reactions in patients undergoing treatment. As a result, efforts are being made to explore strategies that can help mitigate the immunogenicity of the drug. Considering the pharmacological prominence of L-ASNase, this review provides a general description of current biotechnological developments in enzyme engineering strategies and tools adopted for the enhancement of anti-leukemic L-asparaginase.

Key words: L-asparaginase, strategies, immunogenicity, stability, rational design

1. Introduction

Enzymes are highly efficient biocatalysts used commercially for various industrial functions, from clinical approaches to biofuels [1]. Among these, L-asparaginase is a significant enzyme belonging to the class of amidohydrolases (L-ASNase) (EC 3.5.1.1), which accounts for 40% of the global demand for enzymes and one-third of the worldwide requirement for anticancer agents, making it significantly more in demand than other therapeutic enzymes. [2, 3]. It is projected that this growth will continue, with a compound annual growth rate (CAGR) of 16.1% expected between 2022 and 2028.[4]. This enzyme is an important biocatalyst: it catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia [5] and can also hydrolyze glutamine with secondary L-glutaminase activity [6].

Research work into L-ASNase dates back to the 1920s, when the potential of L-ASNase in cancer treatment was first described [7]; however, the approach to L-ASNase can be traced back mainly to the 1960s, when it was observed that guinea pig serum exhibited antitumor activity against lymphoma, both *in vitro* and *in vivo* [8]. Years later, it was established that this enzyme is the antitumor principle acting in the Guinea pig, and that the purified L-ASNase derived from *Escherichia coli* exhibited antitumor activity similar to that of guinea pig serum [8-14]. This discovery paved the way for the successful use of bacterial-derived L-ASNase [12]. Currently, several sources of the enzyme have been found, including bacteria, fungi, algae, birds, plants and animals, excluding humans [15].

L-ASNase is currently used in two main industrial applications which can be seen in **¡Error! No se encuentra el origen de la referencia.**. The primary application of L-

ASNase is as an antileukemic agent in the treatment of acute lymphoblastic leukemia (ALL), which accounts for 80% of pediatric acute leukemias and 20% of leukemias in adults. [16, 17]. There are four approved clinical-grade L-ASNases and correspond to those of bacterial species like L-ASNase from *Erwinia chrysanthemi* (ErA), which is marketed as Erwinase®, and L-ASNases from *Escherichia coli* (EcA), which are found in three formats in their native form (Kidrolase®, Elspar® and Leunase®), as a conjugate with polyethylene glycol (PEG) (Oncaspar® and Sprectrila®) and in their recombinant form [15, 18]; In addition to its biopharmaceutical application, L-ASNase is also utilized in the food industry as a mitigation agent for acrylamide (AA) [19], a potential carcinogen (level 2A) [20]. AA is produced by thermal processing of carbohydrate-rich foods due to the Maillard reaction [3], this occurs in foods such as french fries and roasted coffee beans [21, 22]. A widely used source for food-grade L-ASNase is *Aspergillus oryzae* [15].

One particular drawback that limits the widespread of L-ASNase in therapeutic applications and requires resolution is its high immunogenicity [23, 24]. In some cases, its use creates silent hypersensitivity, which leads to the generation of antidrug antibodies [25]. As a result, numerous strategies have been devised to enhance the efficacy of L-ASNase, including biomolecular and biochemical techniques. For example, mutagenesis, directed evolution, fusion proteins, conjugates with polymers, glycosylations and *in silico* techniques [26, 27]. This review aims to discuss the enzyme engineering strategies and tools utilized to enhance the anti-leukemic L-asparaginase in terms of minimizing immunogenicity and increasing enzyme stability. The article also sheds light on recent advancements in the production of less immunogenic biopharmaceuticals, such as the incorporation of artificial intelligence, encapsulation, and molecular techniques.

2. L-ASNase mechanisms

Unlike healthy cells, leukemia cells exhibit lower gene expression levels of asparagine synthetase (ASNS), which can be attributed to epigenetic regulatory mechanisms such as hypermethylation of the CpG islands of the promoter, or by histone methylation and acetylation [28, 29]. ASNS is responsible for the synthesis of asparagine (ASN) from aspartate [30, 31]. L-asparagine is an important non-essential amino acid for the growth and development of healthy and neoplastic cells by acting on the biosynthesis of proteins. If this amino acid is not available, cell proliferation becomes difficult [32] because cell cycle arrest in the G0/G1 phase is induced. Which in due process, drives the induction of caspase-dependent apoptosis or autophagy by depletion of L-ASN [31, 33]. As the neoplastic cells are unable to synthesize L-ASN, they need an extracellular source of asparagine [3, 5, 34]. Considering the absence of ASNS in certain cancers, such as leukemia and lymphoma [35, 36]; and that depletes ASN from the extracellular medium, leaving cancer cells unable to obtain this non-essential amino acid; is that L-ASNase has emerged as a potential treatment for cancer.

3. Side effects related to L-asparaginase use

The administration of L-ASNase therapy is linked to the occurrence of unfavorable responses in patients who receive the medication. A factor that triggers these drawbacks is the dual activity of L-ASNase (asparaginase and glutaminase), as the starvation of glutamine in the system can cause acute pancreatitis, thrombotic complications and immunosuppression [32, 37, 38]. Glutamine starvation has been described to cause more side effects than treatment as an anticancer [6]. In addition, it has been reported that the

115 catalysis of ASN and its deficit in the body generate side effects, such as nausea, fatigue,
116 diarrhea, vomiting, headaches and abdominal pain [39-41]. These harmful effects arise
117 due to the disruption of protein synthesis [42], as asparagine plays a crucial role in
118 regulating this process by serving as a metabolic controller of the tricarboxylic acid (TCA)
119 cycle and cellular nitrogen supply, which are necessary for the synthesis of non-essential
120 amino acids [43, 44]. It has also been reported, that the deficiency of L-asparagine
121 combined with the accumulation of ammonia in the brain causes depression, anxiety,
122 lethargy, fatigue, etc. [45]. Another source of side effects is related to the prokaryotic
123 origin of the L-ASNases used for the treatment. Because when the enzyme is
124 administered, the body responds by producing antibodies that cause hypersensitivity to
125 the drug or even anaphylaxis [46, 47].

126 These hypersensitivity reactions can also be manifested by silent inactivation of L-
127 ASNase. This is due to the formation of anti-asparaginase antibodies, together with the
128 activity of native protease enzymes present in the blood system, which neutralize the L-
129 ASNase that has been injected into the bloodstream. Which, in due course, reduces
130 efficacy of the enzyme without causing an obvious allergic reaction [48-50]. Clinically, this
131 problem is addressed by changing the bacterial source of L-ASNase. L-ASNase extracted
132 from *E. coli* is considered the first-line therapy in most cases. If severe side effects or
133 hypersensitivity are experienced, the administered enzyme is changed to *Erwinia*-derived
134 L-ASNase [18], however this presents problems such as short half-life (0.65 days in the
135 body), leading to the need for multiple doses [51, 52]. Another solution to the
136 hypersensitivity problems, is to modify the treatment with the PEGylated form of *E. coli*-
137 L-ASNase [53], which compared to others found commercially, provides a half-life in

blood serum 5 times longer than that of *E. coli* [54], which leads to incur in the use of fewer doses. Thus, reducing the development of unwanted antibodies resulting from multiple doses [55]. However, if antibodies to native L-ASNase have already been induced, cross-reactivity with PEGylated L-ASNase may occur [56]. Thus, allergic reactions and antibiotic resistance are increased.

4. Enzyme engineering in L-asparaginase

Recombinant DNA technology is an important strategy to improve protein yield [57]. Recombinant protein production has a significant impact on enzyme commercialization, as all microbial enzymes used are expressed in efficient heterologous expression systems [15]. *E. coli* has become the most preferred host, due to its extensively studied genetic composition, high cell density and simple culture conditions [58].

The most sought-after goal in the production of L-ASNase is to engineer an enzyme with diminished glutaminase activity, reduced immunogenicity, and enhanced stability for use as a biopharmaceutical. The optimization of biological stability is often coupled with the development of enzyme engineering tools based, with focus on strategies for the rational redesign of enzymes, becoming one of the most promising tools to obtain improved enzymes with the desired physical and catalytic properties [59]. Rational redesign is based on providing precise modifications to the amino acid sequence, using detailed knowledge of the structure, function and mechanism of the protein. These modifications are driven by mutagenesis [60], with the aim of de-immunizing the enzyme. Other technique for reducing immunogenicity is the conjugation of the enzyme at exposed sites to prevent antibody formation. Another approach is based on enzyme re-engineering to

overcome the short half-life of L-ASNase by improving its structural stability [61]. This will be discussed in more detail below.

4.1. Site-directed mutagenesis

Site-directed mutagenesis is a technique that falls under the umbrella of "homology-based engineering", which studies the influence of the structure-function relationship, using techniques such as PCR to generate substitutions, deletions and/or point insertions in DNA, in order to design novel, optimized catalytic activities and improved biophysical properties in proteins [62, 63]. It is possible to perform site-directed mutagenesis on enzymes that have a known sequence, obtaining a recombinant protein with improved specificity, stability, activity, solubility [64] and improved L-ASNase properties [15].

Commercially available L-ASNases are not free of glutaminase activity, and may hydrolyze up to 9% of the total enzyme activity [26], which contributes to some of the side effects in the treatment of ALL [65, 66]. Thus, this technique is commonly used to achieve L-ASNases free of glutaminase activity [67, 68]. Table 1 shows some studies where targeted modifications were made in one or more amino acids of the genes encoding L-ASNases from different sources to reduce glutaminase activity and/or increased affinity to ASNs. The targets of these site-directed mutations are conserved residues that interact with glutamine, but are not essential for the asparaginase activity of L-ASNase [6, 59, 69-75].

Eliminating the glutaminase activity of L-ASNase has not been the solely goal of researchers. They have also sought to increase resistance to the medium, either by providing modifications to deliver a longer half-life in the bloodstream or by reducing their

antigenicity. In the study by Sannikova et al. (2016), L-ASNase (WsA) was obtained. Site-directed mutagenesis was performed on V23Q and K24T to attain an enzyme that was stable as well as resistant to trypsin-like proteases. Jianhua, Yujun [76] mutated an amino acid triplet 195RKH197 to 195AAA197 to decrease the major immunogenicity responses caused by L-ASNase obtained from *Wolinella succinogenes* (WsA). After mutation, an asparaginase with lower antigenicity was obtained (see Table 1).

With site-directed mutagenesis, a promising L-ASNase enzyme can be obtained for biopharmaceutical use, since these mutations contribute to decreased glutaminase activity, thereby decreasing immunogenicity when administered in blood serum. However, these mutations need to be complemented with bioinformatics analysis to find the conserved sites of the mutations, as well as further analysis to verify that these mutations do not alter the stability and activity of the enzyme.

4.2. Directed evolution

"Directed evolution" is a strategy, where information concerning the structure and function of the enzyme is not required [77, 78]. It is based on the Darwinian principle of natural selection and performs advanced engineering on the enzymes to adapt and recombine with improved properties at a faster rate [79]. It is based on a random process that takes advantage of the repair limitations of the DNA and polymerases. A polymerase chain reaction (PCR) with an error-prone polymerase, mutated strains or chemical mutagens can be used to create a library of random mutagenic genes to later identify the mutants with improved properties [60, 80]. The process is repeated several times until the desired

trait is achieved. The mutant gene will be cloned in a compatible plasmid and transformed into a strain for the functional expression of the recombinant protein [64].

Libraries of mutant genes have been created for the production of L-ASNase from different sources, where the goal is to improve the enzymatic activity for L-asparagine by changing specific amino acids that intervene in the enzymatic activity and the enzyme substrate bond [81-83]. Table 2 displayes several examples of the directed evolution strategy. Heat-stable proteins have also been designed, since their body-residence time improves and minimizes the immunosuppressant effect. Thus, reducing the therapeutic dose required. It has been observed that in L-ASNase from *Erwinia chrysanthemi* (*ErA*), by means of the stepwise extension process (StEP), a technique for the formation of mutagenic libraries, a heat-stable mutant enzyme was achieved that had a mean inactivation temperature of 55.8°C and a half-life of 159.7 hours, being approximately 60 times higher than the half-life of the native enzyme. [84]. Although this is a strategy used to obtain L-ASNase mutants, it takes time to test whether the mutations have the desired effects on the enzyme, so it would be more appropriate to work with strategies based on the rational design of enzymes with site-directed mutations, where the characteristics of the enzyme are taken into consideration.

4.3. Fusion protein linkers

As stated, a problem associated with the use of bacterial proteins for biopharmaceutical purposes, is their short half-life in blood serum. The primary reason for this is that their small size and cargo make them highly vulnerable to removal through renal filtration. This can be overcome by fusing the enzyme with proteins or domains that have a longer half-

life, such as crystallizable fragment (Fc), transfer fusion (Tf) or albumin fusion protein [15]. Research has already been conducted on the coupling of fusion proteins with L-ASNase. In a study by Guo, Wang [85], a fusion protein composed of EcA and a single-chain variable fragment (scFv). The antibody fraction of the fusion protein was fused to the N-terminus of the enzyme via a linker peptide. This protein had approximately 82% of the enzymatic activity of the native L-ASNase and presented increased stability. Sannikova, Bulushova [86] conducted a study in which they constructed a recombinant variant of was L-ASNase obtained from *Wolinella succinogenes*, which was then fused with heparin to enhance the therapeutic effectiveness of the enzyme. This fusion aimed to increase the biological activation, stabilization, and half-life of the enzyme, with the goal of comparing the efficacy of protein fusion. Tests in mice revealed that the heparin-fused protein had greater therapeutic efficacy than the unfused recombinant enzyme. Thus, fusion protein linkers provide a suitable mean for increasing half-life. Nevertheless, the process of enzyme re-engineering can sometimes lead to a reduction in enzyme activity, as the complex structure of L-ASNase can pose challenges. Successful fusion requires a deep understanding of the three-dimensional structure of the fused protein, as well as bioinformatics analysis for predicting enzyme activity and producing enzymes that are both more stable and therapeutically effective.

4.4. Conjugation with polymers

Another strategy to prolong the half-life of a biological product is PEGylation, which is based on the covalent bonding of bioactive molecules to a poly(ethylene glycol) (PEG) chain [87]. PEG is a polymer formed by ethylene glycol (EG) subunits bound to water molecules. Due to this feature, peptides or proteins conjugated with this polymer display

248 improved solubility and stability [87]. Oncaspar® is a commercially available example of
249 such enzyme; it uses the method of PEG conjugation with free amines, normally in the
250 lysine residues and the N-terminal end. A limitation of this technique is that proteins
251 usually present many lysine residues and, therefore, PEGylation occurs randomly. Which
252 leads to a high degree of polydispersity in the resulting preparations. Thus, producing
253 enzymes with different pharmacokinetic profiles and possibly different intrinsic biological
254 activities [88, 89]. To tackle with these limitations, studies have been designed to improve
255 PEGylation protocols. One such strategy, has focused on site-specific PEGylation,
256 seeking for the production of controlled and standardized PEGylated enzymes. The work
257 by Ramirez-Paz, Saxena [90] was the first to report site-specific PEGylation and the
258 intramolecular cross-linking of L-ASNase subunits in preselected canonical cysteines
259 introduced by mutagenesis in the sites A38 and T263. The advantage of this method is
260 that it keeps the modifications to a minimum, offering the opportunity to direct the
261 immunogenic and potentially proteolytic epitopes in order to conserve the catalytic
262 activity. This PEGylated L-ASNase provided a catalytic activity greater than the native
263 one (210 ± 11 vs. 161 ± 9 U/mg respectively). These results were unexpected as PEG
264 reduces the potency of the drug by restricting the interaction between it and its target [91].
265 Meneguetti, Santos [92] designed an optimized N-terminal PEGylation protocol with
266 monoPEG-ASNase. Their efforts produced an enzyme that was stable for a longer period,
267 than the non-PEGylated enzyme, that displayed resistance plasma proteases and with
268 activity against leukemic cell lines in *in vitro* models. Torres-Obreque, Meneguetti [93]
269 obtained L-ASNase produced by N-terminal PEGylated *Erwinia chrysanthemi* that was
270 more stable at high temperatures and for a more prolonged period. They also compared

it to the *Erwinase* commercial enzyme that lost 93% of its specific activity at two weeks, whereas the PEGylated enzyme remained stable for 20 days. While PEG has been considered a non-immunogenic material with a proven history of safety in humans, it is not free of Hurdles. Recently, anti-PEG antibodies have been foun in both, patients who take PEG conjugates and in healthy individuals [94]. Risk factors of PEG-ASNase reactions have been identified and the effect that it has on the development of antibodies and adverse responses. Reaching the conclusion that 81.5% of patients with an allergic reaction, display antibodies against PEG-ASNase, with anti-PEG being the main antigen [95].

Polymers with greater biocompatibility have been sought to reduce antibody development. One of these is carboxymethyl cextran (CMD), a polymer with biological compatibility. Chahardahcherik, Ashrafi [96] made a conjugate of CMD-L-ASNase, and it was found that the modification increased the specific activity and efficiency of the enzyme. It also showed an increase in half-life and greater resistance to digestion with trypsin. However, further studies in protein engineering and chemical modification with carbohydrate polymers are required because it is a strategy that is being developed to overcome side effects [97-100].

4.5. Glycosylation

Glycosylation is typically accomplished via post-translational modification through an enzymatic process that enables the attachment of glycans to side chains of asparagine (N-glycosylation), serine, or threonine (O-glycosylation) [101]. The production of glycosylated recombinant proteins could improve the effectiveness *in vivo*, optimizing the

biological stability and minimizing hepatic clearance; thus, improving the pharmacokinetic and pharmacodynamic properties of the drug [102]. Protein glycosylation largely depends on the expression system used, since it induces variations in nature and number of glycans added, which can affect the biodistribution of the protein [101]. The glycosylated products are generally produced in mammalian cell lines, which can correctly make complex modifications after the translation into the final bioproduct. In a study by Dantas, Caetano [103], they produced recombinant *E. coli* L-ASNase in mammalian cells in order to produce the glycosylated enzyme. A L-ASNase with a greater molecular weight was obtained by informatic analysis predicted 6 potential glycosylated sites. The data suggests that the L-ASNase obtained in the HEK-293 cell line had optimum relative activity at different pHs and temperatures. However, expression systems based on mammalian cell lines have significant disadvantages. Such as, low protein secretion, easy contamination by viruses, high batch variability, and expensive industrial processes [104].

Another alternative is to use yeast cells for the expression of recombinant glycosylated bioproducts, since they are highly productive and less costly fermentation processes. Nonetheless, the glycosylation pattern is significantly different from the humanized profile obtained from mammalian cells. There is a yeast *Pichia pastoris* which has been modified with genetic engineering techniques in the N-glycosylation pathway allowing the expression of recombinant proteins with mammalian glycosylation [105]. In the study by Lima, Effer [106], a homogenously glycosylated L-ASNase was obtained from *Pichia pastoris* with $Man_5GlcNAc_2$ glucan residues in five potential glycosylation sites. Nevertheless, the glycosylation process hindered the enzymatic activity of recombinant L-ASNase, reaching a value of 3 U/mg, almost 30 times lower than the native enzyme.

316 Effer, Kleingesinds [107] expressed three recombinant enzymes from *D. chriysanthemi*
317 Erwinase expressed in *P. pastoris.* All were enzymatically active and glycosylated, which
318 were able to retain the enzymatic activity of native Erwinase by 3.56%, 68.5% and 11%;
319 they also presented a reduction in the recognition of L-ASNase by anti-Erwinase
320 antibodies. Extracellular expression of L-ASNase was also evaluated with two
321 constructions of the *asnB* gene (with and without His-tag). It was noted that when the His-
322 Tag was absent, protein expression and processes improved, this is due to the His-Tag
323 tail being able to affect the native folding from L-ASNase [108].

324 ### *4.6. In silico* techniques

325 Protein engineering based on analysis of structures and sequences has led to
326 improvements in the properties of enzymes [109], which has increased with the arrival of
327 computational biology and bioinformatics in computer-assisted enzyme modification.
328 Computational protein design has gradually formed a set of systematic methods and their
329 viability has been verified experimentally [110]. Easy access has been granted to predict
330 the effects of the mutations, which facilitates the identification of positive variants [111].
331 Studies on L-ASNase are not exempt from bioinformatics-based tools. It can be seen in
332 the design of L-ASNase to reduce immunogenicity and increase the retention time in
333 circulation, by mapping epitopes and avoiding their recognition of the protein by the
334 immune system [80]. Belén, Lissabet [112] use an *in silico* immune-informatics tool for
335 the prediction of epitopes that contribute to the immunogenicity and allergenicity of *E. coli*
336 (*EcA*) and *Erwinia carotovora* (*EwA*) L-ASNase. It was found that the allele HLA-
337 DRB1*07:01 is associated with a high risk of hypersensitivity and that EcA presents a
338 greater number of epitopes than *EwA*. In addition, Pokrovsky, Kazanov [113] endeavored

to find presumed epitopic regions using a structural and sequential analysis with bioinformatics, comparing five L-ASNases produced by different sources: *W. succinogenes* type II (*WsA*), *Yersinia pseudotubercolosis* type II (*YpA*), *Erwinia carotovora* type II (*EwA), Rhodospirillum rubrum* type I (*RrA*) and *EcA*. Their results revealed nine regions of the enzyme as putative epitopes, where five of these, represent active site loops among the experimentally verified epitopes, the two most reliable contributing to the immunogenicity of L-ASNase are the active site regions, as one is located in the longer N-terminal loop and protrudes far, and the second is located in the C-terminal helix near the loop involved in the active. In the study by Yari, Eslami [114], the conformational B-cell epitopes of Erwinasa were obtained from its three-dimensional structure; two mutants H240A and Q239A were obtained that presented a significant reduction in immunogenicity. Nevertheless, H240A yielded the best result in hydrophobicity, stability and accessibility to the active site. The ASNase enzyme produced by *Pectobacterium carotovorum* (PecA) sought to reduce its immunogenicity by identifying and mutating epitopic regions of B and T CD4+ cells of the enzyme; making the mutations of the epitope peptides produced a 50% reduction in immunogenicity, maintaining their stability in terms of structure and the asparaginase activity [115].

Strategies to reduce glutaminase activity focus on performing *in silico* mutagenesis by means of molecular dynamics, substituting amino acids near the ligand binding site. It was observed that when amino acid ASP96 was replaced by alanine, the glutaminase activity dropped by 30% and also increased the asparaginase activity by 40% in *Pectobacterium carotovorum* [116]. In addition, a model was made of L-ASNase bonded with asparagine or glutamine, where it was detected that the difference between the

interactions occurs with Q59L, which coordinates the main groups but not the side chains of either substrate [6]. A similar strategy was performed on *EcA*, where it was found that mutant V27T is a good candidate to reduce glutaminase activity without affecting the stability of the enzyme [117].

The resistance to proteases bound to amino acid Asn 24 can also be studied. With computational protein engineering tools using a genetic algorithm in combination with molecular dynamics, it was predicted that the mutants in N24T and N24A of L-ASNase had greater activity than the native one and that mutant N24A/R195S had reduced its glutaminase activity [68].

The *in silico* design of proteins allows obtaining potential therapeutic proteins, while aiding to reduce experiments, since the analyzes are carried out previously. Contributing positively to the rational design of enzymes.

5. Conclusions and future prospects

L-ASNase is an important industrial enzyme responsible for the hydrolysis of L-asparagine, a non-essential amino acid crucial for the development of neoplastic cells. This enzyme is produced naturally by a large number of microorganisms and species; those currently used in the pharmaceutical industry are produced by *E. coli* and *E. chrysanthemi*. However, both enzymes face the challenge of immunogenicity and resistance due to the development of antibodies, which makes their application difficult. Due to this fact, protein engineering strategies have been designed that contribute to the reduction of their side effects and increase their stability for use as a biopharmaceutical (such as those discussed in this review article). However, there are still challenges to

384 overcome fthat require further research in the field of protein engineering, such as further
385 enhancing *in silico* analyses. Which are promising tools for predicting epitope regions and
386 modelling enzyme structure, allowing enzyme stability to be predicted, optimizing the time
387 and costs associated with *in vivo* analyses.

388 The trend in enzyme de-immunization is towards the development of increasingly
389 sophisticated algorithms and programs that help to reliably-predict epitopes and suggest
390 possible mutations that are not detrimental to enzyme activity. The use of artificial
391 intelligence is the gateway to efficient enzyme de-immunisation, such as ABCPred which
392 uses recurrent neural networks for B-cell epitope identification and achieves 67%
393 sensitivity, 65% specificity and 66% prediction [118], or ElliPro, a web-based tool for
394 antibody epitope prediction and visualization [119]. And the development of more
395 sophisticated methods using neural networks for improved prediction of epitopes and
396 other similar surface patches, by exploiting features derived from antigens and their
397 related antibody structures, combined with statistical and machine learning algorithms
398 [120]. While these strategies are innovative, they are not yet perfect in prediction, as these
399 lack extensive training data.

400 Another predominant approach in cancer drug development is the use of encapsulation
401 technologies. This field seeks "smart" delivery, where drugs can be delivered with
402 precision, control and stability. In addition, the use of nanotechnology has been observed
403 to provide improvements in drug immunogenicity [121]. Erythrocytes have been used for
404 ASNase encapsulation because they are completely biodegradable and provide
405 protection for the enzyme by preventing an immune response [122, 123]. Encapsulation
406 techniques that do not alter the membrane and structure of red blood cells so that they

cannot be recognized and eliminated by the immune system, are currently being used [123, 124]. In the study [122] a new method of protein encapsulation in red blood cells by cell penetrating peptide (CPP) was presented, where L-ASNase was conjugated to CPP by disulfide bonding and showed an improvement in survival of 44% compared to standard hypotonic loaded red blood cells with a survival of 16.7%, this is a promising method for reducing immunogenicity. However, it still has limitations due to the lack of knowledge and experience in the clinical setting to handle the RBC loading procedure. In addition, these red cells are not yet suitable for clinical use. Another proposed drug delivery alternative is the use of exosomes, which are nanometer-sized vesicles used in drug delivery for various types of treatments. However, there are still limitations in the use of this type of delivery due to difficulties in isolation, efficient drug loading approaches and the regulations that must be met for them to be approved for clinical use [125].

The use of genetic engineering techniques has allowed the development of several modified versions of L-asparaginases that display improved pharmaceutical properties in comparison to the original enzymes. The latest progress in programmable nucleases such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas have made gene editing a practical reality in clinical applications. These techniques have demonstrated success in decreasing the immune system response to certain enzymes, enhancing their target specificity and stability [126]. Recently, the use of CRISPR-Cas9 for the modification of humanized n-glycosylation of recombinant proteins using microalgae has been reported, with the aim of reducing immunological effects [127, 128].

To conclude, in order to advance in the efficient de-immunization of L-asparaginase without affecting its stability and enzymatic activity, advances in epitope recognition and epitope coating tools are required. The trend is towards the development of more robust algorithms, less immunogenic L-ASNase encapsulation strategies and the implementation of new advances in genetic engineering.

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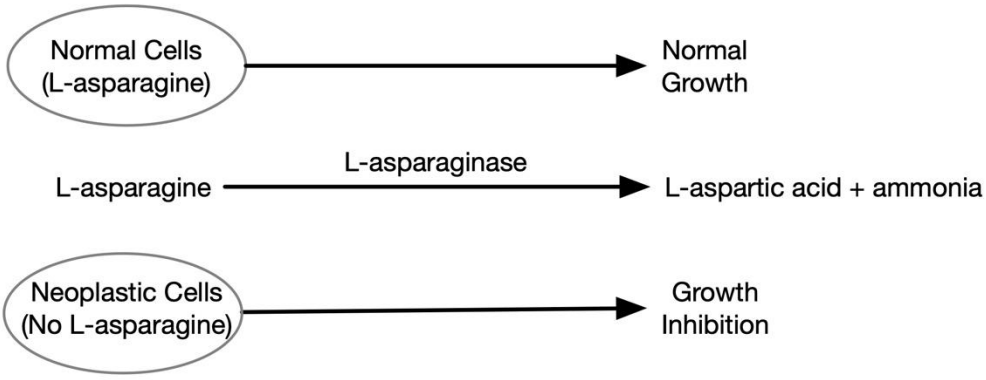
L-asparaginase as anti-carcinogenic agent



L-asparaginase as anti-acrylamide agent

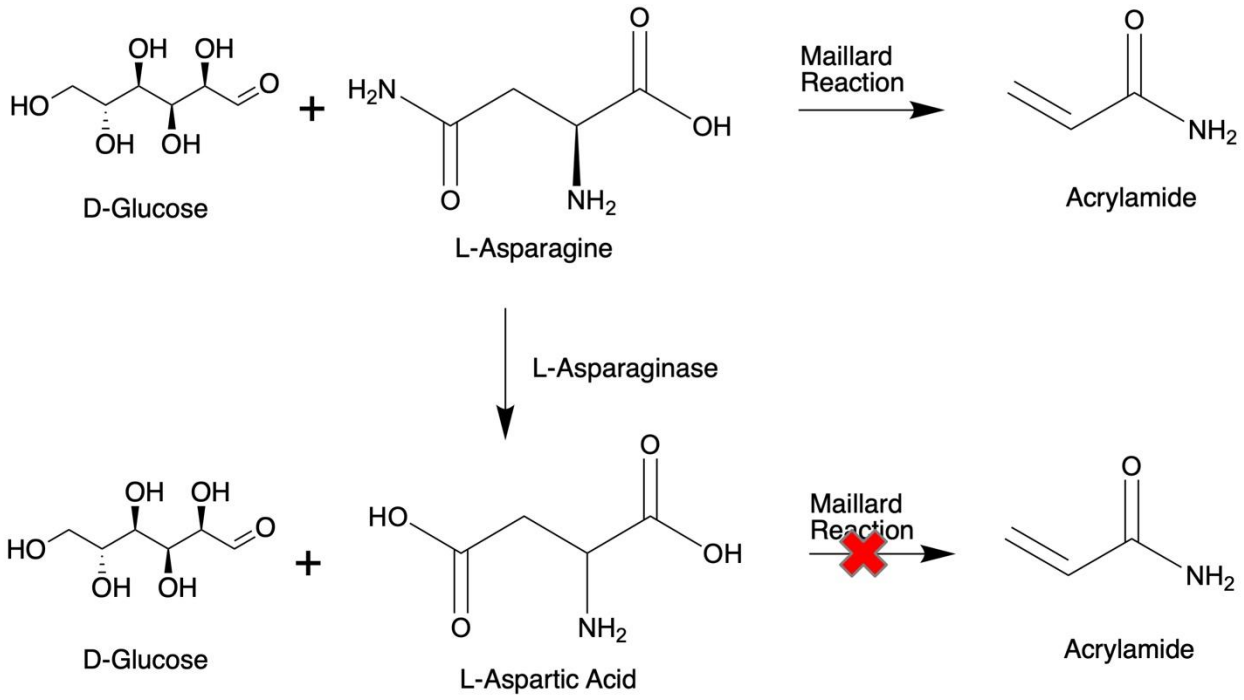


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Figure 1. L-ASNase mechanism used at industrial level as biopharmaceutical and acrylamide mitigation agent.

809 Table 1. L-ASNases modified by site-directed mutations

Host	Gene	Mutation	Results	Reference
E. coli	*ansB*	Q59L	80% asparaginase activity and 0% glutaminase activity	[6]
Bacillus licheniformis	*ansA3*	G238N, E232A, Q112H and D103V	It was observed that the mutations in G238N, E232A and Q112H showed a loss of activity whereas D103V has greater activity and affinity for asparagine.	[59]
E. coli	*ansB*	Y176F, Y176S	They exhibit much lower glutaminase activity than the native one	[69]
E. coli	*ansB*	Y176F, Y176S, K288S, K288S/Y176F, K288R, K288R/Y176F	Y176F and Y176S showed reduced glutaminase activity, K288S/Y176F reduced antigenicity in comparison with the wild one.	[70]
Pyrococcus furiosus	*PfA*	K274E, T53Q, T53Q/K274E	No mutant enzyme presented glutaminase activity with greater cytotoxic activity in human cells compared to the native one.	[71]
Wolinella succinogenes	*WsA*	V23Q and K24T	With resistance to trypsin-like proteases	[86]
Erwinia chrysanthemi	*ErA*	N41D, N281D, N41D/N281D	N41D and N281D conserve their specific activity and the double mutant increases its specific activity towards asparagine.	[72]
Helicobacter pylor	*HpA*	T16D, T95E, M121C, T169M, M121C/T169M	T16D and T95E inactivate the glutaminase and asparaginase activity of the enzyme. M121C and T169M present reduced catalytic activities. M121C/T169M does not generate cytotoxic effects and does not present glutaminase activity.	[73]
Erwinia chrysanthemi	*ErA*	E63Q	It makes the correct positioning of L-glutamine difficult, but not of L-asparagine, given a 25-fold reduction in glutaminase activity	[74, 75]

E. coli	*EcA*	195RKH197 changed for 195AAA197	Antigenicity was reduced because the mutated residues contributed strongly to the antigenicity	[76]

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811 Table 2. *L-ASNases modified by directed evolution*

Host	Gene	Technique	Mutation	Results	Reference
Bacillus megaterium H-1	*ansA*	Error-prone polymerase chain reaction (EP-PCR)	X3D12, X4E7, X5G11, D9B, DD12G	They showed enzymatic activity towards improved L-asparagine, where X2E7, D9B and DD12G exhibited an increase of 8.75, 20.22 and 21.33 times more than the native one.	[81]
Erwinia chrysanthemi	*ErA*	Stepwise extension process (StEP)	D133V	A more heat-stable mutant enzyme was obtained, reaching a mean inactivation temperature (T_m) of 55.8°C with a half-life of 159.7 h	[84]
Erwinia carotovora	*EcA*	StEP	L71I	Lack of glutaminase activity and its activity for asparaginase is duplicated, the changes in the amino acid can alter the conformation of the residues of the active site	[82]
Bacillus subtilis 168	-	EP-PCR	Promotor P43 pP43NMK	L-ASNase activity increased up to 51.13 U/ml.	[83]
Human	*hASNase 1*	EP-PCR and combinatorial active-site saturation test (CAST)	M22/R23, D84/S86, H114/G115, A142/Q143/V144, A191/R192, T118/F121, A91/C95/T99, R23/E25/L26	Active variants were not selected using this method	[99]

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