ENGINEERING AND ENCAPSULATION OF L-ASPARAGINASE IN HUMAN α2-MACROGLOBULIN: A PROTEIN-COMPLEX FOR THE ACUTE LYMPHOBLASTIC LEUKEMIA TREATMENT

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ABSTRACT

L-asparaginase (L-ASNase: EC 3.5.1.1) is a first-line medicine approved 40 years ago for the treatment of acute lymphoblastic leukemia (ALL), a type of blood cancer that commonly affects children. However, despite the scientifically proven success in the treatment of ALL, intrinsic side-effects still need to be overcome, such as: hypersensitivity and immunological reactions. Hence, there is a current need for strategies to circumvent L-ASNase shortcomings such as the exploration of new enzyme sources as well as the development of novel recombinant biobetter L-ASNases. For the last approach, the use of protein engineering techniques and different encapsulations methods have been applied to create more stable variants with less immunogenic potential. In this report we developed through site-direct mutagenesis a new triple-mutant L-asparaginase (3M) that shows better biochemical features than wild-type L-ASNase (WT). Besides that, human α2 -macroglobulin (α2M) was used as medicinal carrier for our 3M L-ASNase. We demonstrate that under appropriate conditions, the interaction between 3M and α2M was viable to improve thermostability, proteolytic resistance, and immunological recognition while preserving the cytotoxicity activity against MOLT-4 leukemic cells. Therefore, these results lead us to propose the human α2-macroglobulin as a general medicinal carrier for incorporation of other enzymes used as biological drugs.

Keywords: L-asparaginase. α2-macroglobulin. Enzyme encapsulation. Protein engineering. Acute lymphoblastic leukemia.

1 INTRODUCTION

L-asparaginase (L-ASNase: EC 3.5.1.1) is a first-line medicine approved 40 years ago for the treatment of acute lymphoblastic leukemia (ALL), a type of blood cancer that commonly affects children.[1] However, despite the scientifically proven success in the treatment of ALL, intrinsic side-effects still need to be overcome, such as: hypersensitivity and immunological reactions.[2] Besides that, the action of human proteases (cathepsin B and asparaginyl endopeptidase) against the biopharmaceutical decreases the enzyme stability while increasing the antibody production and hypersensitivity reactions. Hence, there is a current need for strategies to overcome L-ASNase shortcomings such as the exploration of new enzyme sources as well as the development of novel biobetter L-ASNases. For the last approach, the use of protein engineering techniques and different encapsulations methods have been applied to create more stable variants with less immunogenic potential.[3]

Human α2-macroglobulin (α2M - Mr ~ 720,000) is a homotetramer enzyme consisting of four subunits of approximately Mr ~ 180,000 each.[4] This enzyme is abundant in the human blood (~ 4 mg/mL) and is responsible for the blood proteinases inhibition through a "trapping" mechanism in which proteinase molecules are confined within a α2-macroglobulin. The contained proteinase continues to show enzymatic activity against small substrates until the complex (α2-macroglobulin-proteinase) is degraded by macrophages. This degradation occurs after the exposure of a small peptide chain that functions as a recognition signal for macrophages. Some works has been demonstrated that α2-macroglobulin can encapsulated no-proteinase enzyme after modification and thus, function as a unique medicinal carrier.[5]

In this report we developed through site-direct mutagenesis a new triple-mutant L-asparaginase (3M) that shows better biochemical features than wild-type L-ASNase (WT). Besides that, α2-macroglobulin was used as medicinal carrier for our 3M L-asparaginase and the protein-complex formed was evaluated for enzyme activity retention, thermal stability, resistance against human proteases and "in vitro" immunological response. In summary, both free and α2-macroglobulin-3M showed to be biobetters L-asparaginase compared to WT-enzyme bringing alternative biopharmaceutical to treat ALL with less side-effects.

2 MATERIAL & METHODS

Gene and vector information

The gene with optimized codons for wild-type *Escherichia coli* L-asparaginase (EcAII - *asnB*) was synthesized by GeneScript (Piscataway, New Jersey, USA) and used as template to create the triple mutant (3M: N24S/P40S/S206C) by site-direct mutagenesis using the Quikchange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California, USA).

Then, the plasmids of native (WT) and triple mutant (3M) enzyme were cloned on *E. coli* DH5α. After recovery of plasmids, they were used to transform the electrocompetent *E. coli* BL21 (DE3) for the enzymes production.

Enzyme production and purification

The lysogenic broth medium (LB) with carbenicillin (50 µg/mL) was used for enzyme production. The culture condition was established following the protocol described by Rodrigues et. al.[6] After fermentation step, the cells were harvested at 4000 *g*-force for 20 minutes at 4°C and submitted to osmotic shock to achieve enzymes from periplasmic fractions. The buffered periplasmic fractions were purified through two chromatographic steps: 1. Weak anion exchange chromatography column (HiTrap™ DEAE FF 5 mL, GE Healthcare Life Sciences, Chicago, Illinois, USA) and 2. Size exclusion chromatography (SEC, column Superdex 200 Increase 10/300 GL, GE Life Sciences) coupled to AKTA™ Purifier (GE Healthcare Life Sciences). The purity of enzymes was estimated by SDS-PAGE (14%) and the protein concentration was determined by absorbance at 280 nm, using the molar extinction coefficient of ε= 23,505 M-1cm-1 and molecular weight (MW) of 34.5 kDa.

Association of Triple mutant L-asparaginase and human α2-macroglobulin

The human α2-macroglobulin (α2M) was purchased from Sigma-Aldrich. The α2-macroglobulin activation was carried out by incubating the enzyme (2-6 mg/mL) with 0.2 M NH_4HCO_3 (pH adjusted to 8.5) for 60 min at room temperature. Triple mutant L-asparaginase (3M) was incorporated into NH_4HCO_3-treated human α2-macroglobulin as previously described by Grøn and Pizzo.[5] Briefly, 3M in 50 mM Tris/HCl buffer pH 8.2 was added at a 40-fold molar excess to activated α2-macroglobulin and incubated overnight at 37°C. No associated L-asparaginase was separated from α2-macroglobulin using a size exclusion chromatography.

Enzymes biochemical characterization

Asparaginase activity was determined using the Nessler reagent (Merck-Millipore). The specific activity is expressed as U (1 unit corresponds to 1 µmol of ammonia released per minute) per milligram of pure protein. Optimal temperature (range from 15 ºC to 65 ºC) and pH (range from 4.0 to 11.0) were determinated as well as the enzymes thermostability applying the method adapted from Cabral et al.[7] The half-life, thermal deactivation constant and thermal stability factor for L-asparaginases were also estimated. Proteolysis resistance against human proteases cathepsin B (CTSB) and asparaginyl endopeptidase (AEP) of ASNase was measured by residual activity after an incubation period of 4 hours at 37 ºC with proteases.[8]

Immunogenic assay and *in vitro* cytotoxicity

The presence of anti-asparaginase antibodies was determined by the enzyme linked immunosorbent assay (ELISA), as described by Zenatti et al.[9] The *in vitro* cytotoxicity was performed using MOLT-4 as acute lymphoblastic leukemia cell line which was obtained from Rio de Janeiro Cell Bank (BCRJ-Brazil). Four different enzyme concentrations were studied using 2×10^4 cells/well for 72 hours, at 37 ºC and 5% CO_2. Next, 0.5 mg/mL of MTT was added and incubated for 3 hours at the same conditions. The absorbance was measured at 597 nm in a spectrophotometer (Spectra Max M2 – Molecular Devices).

3 RESULTS & DISCUSSION

The found results are summarized in the Table 1. Site-direct mutagenesis has been applied to obtain new biobetter L-asparaginase.[1] Herein, we used the gene from *E. coli* responsible for the commercial L-asparaginase production as a template to create the triple mutant L-ASNase: N24S/P40S/S206C. The Table 1 shows that some parameters analyzed were improved after mutation (thermostability and protease resistance) and others did not showed alterations. These results confirm the potential of protein engineering as excellent tool to improve the biochemical features of enzymes used as biological drugs.

Table 1. Comparison of biochemical properties of triple mutant L-asparaginase (3M) associated with human macroglobulin and the free enzymes (3M and WT).

PARAMETERS	WT	3M	3M-Macroglobulin
Encapsulation Rate (%)	-	-	25.0
Optimum Temperature (°C)	55.0	55.0	55.0
Optimum pH	7.50	7.50	7.50
K_d (hs) 37°C	0.121	1.029	1.070
K_d (hs) 50°C	0.060	0.525	0.515
$T_{1/2}$ (hs) 37°C	7.98	12.60	18.50
$T_{1/2}$ (hs) 50°C	0.67	1.29	1.98
Stabilization Factor - 37°C	-	1.50	1.51
Stabilization Factor - 50°C	-	2.00	1.63
Resistance - Cathepsin B (U/mg)	29.55	34.55	41.34
Resistance - AEP (U/mg)	21.38	38.91	44.25
MOLT-4 - IC_{50} (U/mL)	0.0011	0.0012	0.0012
Elisa assay (ABS 492 nm)	0.941	0.801	0.500

WT= Wild type L-asparaginase; 3M= L-asparaginase triple mutant; 3M- Macroglobulin = Macroglobulin-associated triple mutant L-asparaginase; K_d= Thermal deactivation constant; $T_{1/2}$= Half-life time; AEP= asparaginyl endopeptidase.

With the intention of further improving the critical characteristics for the using of L-asparaginase as biopharmaceutical, we evaluated the use of human macroglobulin as a medicinal carrier. After appropriated activation of macroglobulin, we produced a new protein-complex through the association of 3M and α2M. The encapsulation rate was around 25% and the influence of the main factors responsible for the sides effects during ALL treatment were reduced – Table 1. Beyond the improvement of thermostability and half-life time, the encapsulated L-ASNase retained 58.5% and 61.2% of enzyme activity after 4 hours of incubation in the presence of cathepsin B and asparaginyl endopeptidase, respectively. The WT and 3M enzyme showed less proteolytic resistance against the same human proteases. Besides that, the ELISA assay for the protein-complex indicates a lower potential in eliciting the immune response compared to wild-type L-asparaginase and free 3M enzyme. Finally, *in vitro* cytotoxicity was carried out using MOLT-4 as leukemic cell line and there was no difference in the values of IC_{50} – Figure 1, showing that encapsulation was not detrimental to 3M ASNase antitumor activity.

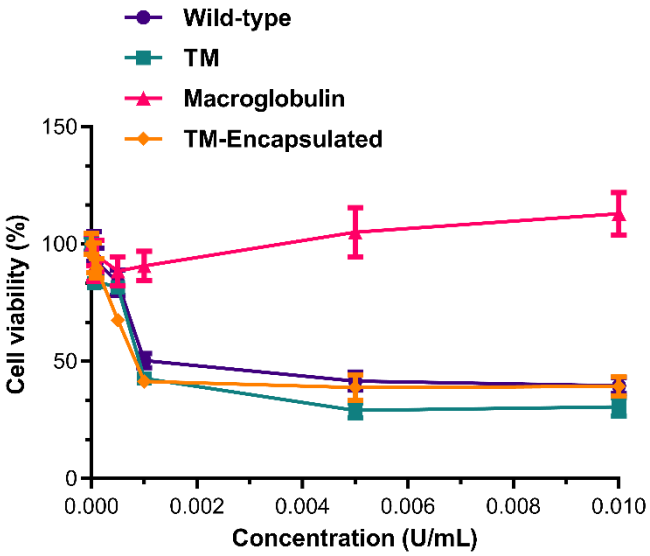

Figure 1. Graph for the *in vitro* cytotoxicity assay of WT, 3M and macroglobulin-encapsulated 3M L-asparaginases using the MOLT-4 leukemic cell line. The assay was carried out in analytical triplicate, with biological triplicate. . The data is presented as the mean ± standard error of the mean (SEM). The IC_{50} is represented by the mean ± standard deviation (U/mL).

4 CONCLUSION

In this study were evaluated two approaches to improve the use of L-asparaginase as biological drug applied in the acute lymphoblastic leukemia treatment: protein engineering and protein encapsulation. The triple mutant L-asparaginase (3M) obtained by site-direct mutagenesis showed improved features compared to wild-type asparaginase: improved thermostability and human protease resistance. Human α2-macroglobulin is a tetrameric enzyme showing a molecular weight of 718 kDa that is highly abundant in human plasma. We demonstrate here that under appropriate conditions, the interaction between 3M and human α2-macroglobulin (α2M) was viable to improve thermostability, proteolytic resistance, immunological response while preserve the cytotoxicity activity against MOLT-4 leukemic cells. Therefore, these results lead us to propose the human α2-macroglobulin as a general medicinal carrier for incorporation of other enzymes used as biological drugs.

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