POLYMERS AND AMINO ACIDS AS ADDITIVES FOR THE STABILIZATION OF THE BIOPHARMACEUTICAL L-ASPARAGINASE

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ABSTRACT

Biopharmaceuticals are essential in treating complex diseases, including certain cancers and autoimmune conditions. However, their high cost and low stability limit their access in underprivileged regions, where even low-cost biologics such as vaccines and insulin are challenging to preserve due to inadequate refrigeration conditions. With this in mind, this study focused on finding additives that allow the preservation of the enzyme L-asparaginase (an antileukemic biopharmaceutical) without refrigeration, aiming to reduce storage and transportation costs. We tested sugars, surfactants, and biocompatible polymers for their ability to maintain the enzyme's catalytic activity under various temperature and time conditions. Polyethylene glycols (PEG) and propylene glycols (PPG) of low molecular weight and leucine stood out for significantly preserving L-asparaginase activity at 55 °C for one hour. Furthermore, by adding leucine and PEG-200, about 40% of the enzymatic activity was maintained after 20 days at 20 °C. These results suggest that such additives can preserve L-ASNase enzyme activity at high temperatures and extend the shelf life of biopharmaceuticals like L-asparaginase, facilitating their distribution and access.

Keywords: pharmaceutical formulations, L-asparaginase, biopharmaceuticals, enzymes, protein stability

1 INTRODUCTION

Advances in biotechnology have led to the development of a series of biomolecules with medical applications, enabling the treatment and increased survival of patients with conditions previously considered incurable, such as certain types of cancer and autoimmune diseases. For example, introducing the biopharmaceutical L-asparaginase (L-ASNase) to treat acute lymphoblastic leukemia (ALL) increased the survival rate five years after the disease diagnosis of children with ALL from 10 to 90%.[1] However, despite their great potential, these biological products are still inaccessible to many disadvantaged communities and low-income countries due to their high prices and low stability, which complicates their distribution, storage, and handling.[2]

The stability of biomolecules is critical for transporting and storing biopharmaceuticals, which ideally should have universal coverage. The Global Alliance for Vaccines and Immunization estimates that only 10% of health centers in the poorest countries have a reliable electrical grid to preserve biopharmaceuticals.[3] An example of a biopharmaceutical that presents distribution and application problems due to their thermal instability is L-ASNase. Currently, lyophilized L-ASNase should be stored between 2 and 8 °C, and the reconstituted solution must be discarded after 8 hours or earlier in case of cloudiness, generating difficulties, losses, and risks in its transportation, storage, and application.[4] Therefore, increasing the stability of biomolecules is essential to expand the applications and access to biological medicines like L-ASNase.

Among the substances with demonstrated potential to stabilize biological products, compounds such as salts and organic acids, amino acids, carbohydrates, surfactants, and biocompatible polymers are classes already used to stabilize and enhance the use of vaccines.[5] However, the application of these additives for other biopharmaceuticals is still limited and low study by researchers.

Hence, this research aimed to find additives that allow the thermal preservation of protein-based biopharmaceuticals (particularly L-ASNase), facilitating their transport and contributing to universal access to safe and lower-cost biotechnological medical products. To this end, we selected different classes of compounds already used in vaccines, such as amino acids, surfactants, polymers, and carbohydrates, and evaluated their potential to increase the thermal stability and long-term room temperature stability of L-ASNase.

2 MATERIAL & METHODS

Pure L-ASNase from *Escherichia coli* was acquired commercially and prepared with phosphate buffer pH 7.4 to simulate physiological pH.

In the first experiment, we evaluated the interference of different classes of compounds (amino acids, proteins, carbohydrates, surfactants, and polymers) at concentrations ranging from 1 to 500 mM on Nessler's reaction, the selected method for the quantification of L-ASNase through hydrolysis of L-asparagine. We also assessed the interference of amino acids and proteins in the quantification of L-ASNase activity. We selected the conditions that did not interfere with Nessler's reaction or L-ASNase

activity determination for further investigation. Moreover, we evaluated the thermal stability of the enzyme at different times and temperatures to select a condition with 40 to 60 % activity loss for the protection studies.

After selecting the compounds and environmental parameters, we determined if low concentrations of polymers, non-reducing carbohydrates, and amino acids preserved the activity of L-ASNase after one hour at 55 °C. Then, we choose the best conditions to study the maintenance of L-ASNase activity in the long term with no refrigeration (40 days at 20 °C).

Finally, we also assessed the impact of the dilute solutions of polymers and amino acids on the activity of L-ASNase from another source (raw extract of L-ASNase from *Aliivibrio fischeri* expressed by the bacteria *Bacillus subtilis*) to evaluate the potential of the additives with enzymes from other origins.

3 RESULTS & DISCUSSION

The first set of experiments investigated the compatibility of various compounds with the Nessler reaction for ammonium ($[NH_4]^+$) quantification, a method for assessing L-ASNase enzymatic activity by measuring ammonia produced from asparagine hydrolysis. Despite the cost-effectiveness and sensitivity of the Nessler reaction, this method is prone to interference by substances that affect the reagent or alter the reaction's pH. Testing identified that reducing sugars, surfactants, and high molecular weight polymers interfered with ammonia quantification, whereas non-reducing sugars, certain amino acids, bovine serum albumin (BSA), and low molecular weight polymers showed minimal interference.

Further tests evaluated the interference of amino acids with *E. coli* L-ASNase activity at 25°C, revealing significant effects only at higher concentrations for proline, glycine, and leucine. All BSA concentrations tested altered the quantification of L-ASNase activity when compared with the control.

The study also assessed the thermal stability of *E. coli* L-ASNase, noting its activity preservation up to 120 hours at 25°C, with rapid decline at higher temperatures. Given the activity loss from 40 to 60 % at 55°C within an hour, we chose this condition to test the effect of the different substances on L-ASNase thermal stability.

For the thermal protection studies, results showed the evaluated polymers protected *E. coli* L-ASNase activity at specific concentrations, with lower molecular weight polymers like PEG-200 and PEG-300 requiring higher concentrations for protection. Interestingly, PPG-400 demonstrated protection at lower concentrations than PEG-400, potentially due to its hydrophobic nature enhancing its interactions with L-ASNase. The best results are presented in **Figure 1**.

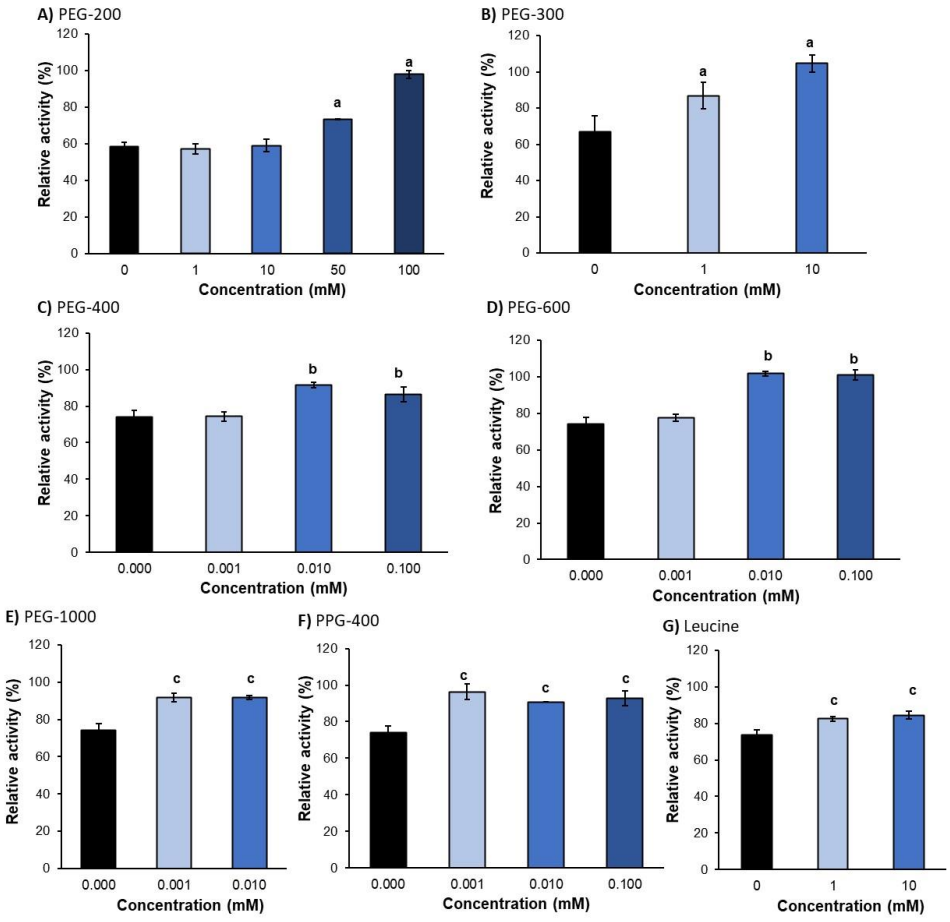

Figure 1. Relative *E. coli* L-ANSase activity (1 U/mL) in different concentrations of polymers and leucine after 1 h at 55 °C. a) Different to control, b) different to all groups, c) different to control and 1 mM (One-Way ANOVA with post-test of Holm-Sidak, $p < 0.05$).

We also assessed the impact of dilute solutions of polymers and amino acids on the activity of L-ASNase from *A. fischeri* expressed by *B. subtilis* to evaluate the potential of using the additives for other enzyme variants. Although there were variations compared to the *E. coli* L-ASNase results, which could be also due to a lower purity of the *A. fischeri* enzyme, the polymers overall also improved the thermal stability of the *A. fischeri* L-ASNase

We selected the conditions that improved the thermal stability of the enzyme to evaluate the effect of polymers and leucine on the preservation of *E. coli* L-ASNase in the long term (40 days) with no refrigeration. At 0 h, the polymers and leucine (concentrations varying from 0.01 to 100 mM) had no impact on the enzyme activity compared with the control in phosphate buffer. However, after six days at 20 °C, while the control lost half of its activity, leucine fully preserved L-ASNase activity. Moreover, the polymers PEG-200, PEG-1000, and PPG-400 enhanced the preservation of L-ASNase activity, while PEG-300, PEG-400, and PEG-600 impaired it. PEG-1000, PPG-400, and leucine partially preserved the activity of L-ASNase for up to 20 days at room temperature, but only leucine maintained 15 % of the enzyme's initial activity after 40 days. The results for 6 and 20 days are shown in **Figure 2**.

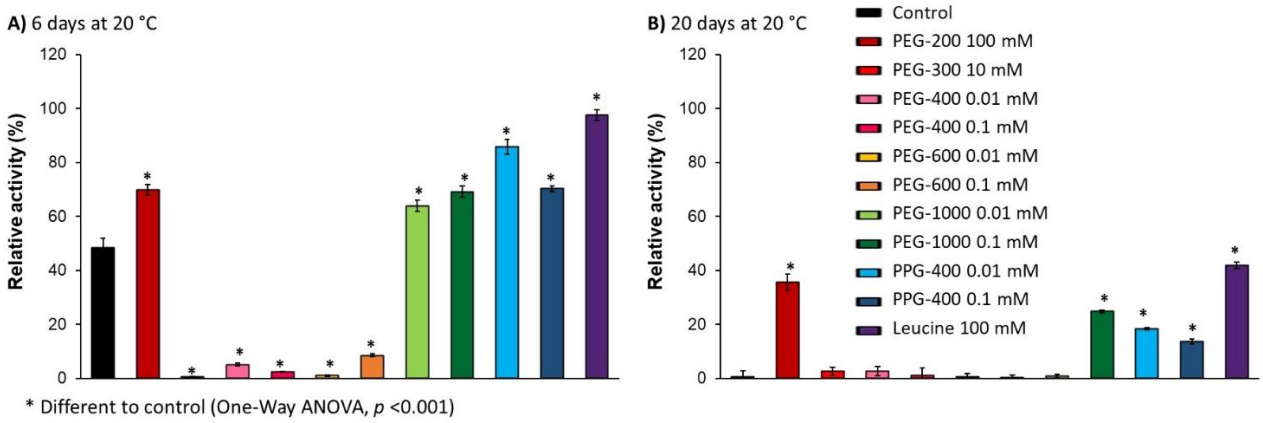

Figure 2. Relative *E. coli* L-ANSase activity (1 U/mL) in different concentrations of polymers and amino acid after **A)** 6 days and **B)** 20 days at 20 °C.

Conclusively, polymers can enhance the thermal stability and long-term stability at room temperature of biopharmaceuticals like L-ASNase, suggesting the feasibility of using traditional vaccine excipients to improve the preservation of other biopharmaceutical classes, including therapeutic enzymes.

4 CONCLUSION

This study demonstrated that dilute aqueous solutions of PEG and PPG of low molecular weight and leucine can significantly preserve the catalytic activity of L-ASNase after thermal stress. Furthermore, leucine can completely preserve the activity of the enzyme after 6 days of incubation at room temperature, while L-ASNase loses half of its activity in the phosphate buffer for the same condition. Therefore, polymers can be used as additives to increase the thermal stability and long-term room temperature stability of biopharmaceuticals such as L-ASNase, having the potential to improve and expand their application and distribution. It is important to highlight that according to the enzyme source, different interactions with the excipients can be obtained due to the arrangement of amino acids that compose the molecule. Therefore, this study demonstrates that it is possible to apply traditional vaccine excipients to enhance the stability of other classes of biopharmaceuticals.

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