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# INFLUENCE OF COSSOLVENTS ON L-ASPARAGINASE CONJUGATION WITH POLYETHYLENE GLYCOL

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## ABSTRACT

Acute Lymphoblastic Leukemia is the most common cancer in children, and the enzyme L-asparaginase is the main drug used in its treatment. However, it presents some problems such as elimination by the immune system or renal excretion. An alternative is the conjugation of polyethylene glycol to the enzyme, however, the reaction has low yield, requiring an excess of polymer to obtain a greater number of conjugates, which increases costs and the difficulty of purification steps. Thus, this work aimed to study the influence of cosolvents acetonitrile, DMSO, ethanol, and methanol on the conjugation reaction to obtain a conjugate with the highest possible number of polymeric chains per enzyme molecule. The enzyme was produced from the cultivation of a strain of *Escherichia coli* BL21 pET26b/ans and purified using immobilized metal affinity chromatography and size exclusion chromatography. Conjugation occurred in the presence of all solvents, and aiming at the objective of the work, DMSO appears to be the most promising, due to the higher intensity of protein conjugate bands in different size ranges.

**Keywords:** L-asparaginase. Pegylation. Polyethylene glycol. Cosolvents.

## 1 INTRODUCTION

The enzyme L-asparaginase (ASNase) has been used for over 30 years in the treatment of Acute Lymphoblastic Leukemia and in the treatment of non-Hodgkin lymphoma [1-3]. It possesses different characteristics depending on the organism that produces it, such as variations in immune system response and variations in the level of L-glutaminase activity that the enzyme also possesses [4-8]. Therefore, there is a constant search for new enzyme sources, and with this in mind, the Bioprocess Laboratory PEQ/COPPE/UFRJ has been studying recombinant ASNase from the bacterium *Zymomonas mobilis* in *Escherichia coli* for several years [9-13].

The enzyme produced through biotechnological processes presents some issues (rapid elimination from the body, immunogenicity, physical and chemical instability, enzymatic degradation) that can be circumvented through its conjugation to polyethylene glycol (PEG) via the formation of a covalent bond[14]. However, the high cost of producing recombinant proteins, combined with the additional cost caused by protein conjugation to PEG, necessitates high purity and high yield for the development of new PEG-protein conjugates[15].

Therefore, continuing the work carried out by the laboratory and considering that there are no records in the literature of the study of conjugation of recombinant L-asparaginase from *Z. mobilis* to PEG, this work aims to investigate the conjugation reaction with the goal of obtaining an ASNase-PEG conjugate with high yield and satisfactory biological activity.

## 2 MATERIAL & METHODS

**Production and separation of ASNase.** The microorganism used to produce L-asparaginase was *E. coli* BL21 pET26b/ans12. For the expression of ASNase, a pre-inoculum was prepared with 100 mL of LB medium (Luria Bertani Broth); 2.5 mL of 40% (w/v) glucose; 100µL of 50 mg/mL kanamycin; and 100 µL of bacterial suspension preserved in 50% (w/v) glycerol, kept in an ultra-freezer at -80 °C[11, 13]. The pre-inoculum was incubated for 8 hours at 37 °C and 200 rpm. After the growth period, 100 mL of the pre-inoculum was transferred to a 2 L Erlenmeyer flask containing 1 L of LB medium, 1 mL of glycerol (60% w/v), 0.5 mL of glucose (10% w/v), 2.5 mL of lactose (8% w/v), and 200 µL of 50 mg/mL kanamycin. The cultivation was carried out for 16 hours at 37 °C and 200 rpm. At the end of the 16 hours, the culture medium containing the cells was centrifuged at 4 °C. The cells were resuspended in 400 mL of 66 mM phosphate buffer, pH 8, and subjected to the cell disruption step using a high-pressure homogenizer. A pressure of 300 bar was applied, and the sample passed through the equipment 4 times. After cell disruption, the cell suspension was centrifuged for 25 minutes at 7500 rpm and 4°C and the supernatant was collected for the purification step.

**Purification of ASNase.** The supernatant was subjected to affinity chromatography using a 5 mL HisTrap column. Column equilibration and washing steps were performed with 66 mM phosphate, 0.5 M NaCl, and 60 mM imidazole. The enzyme was eluted with 66 mM phosphate, 0.5 M NaCl, and 300 mM imidazole. To remove imidazole from the sample, size exclusion chromatography was performed, in which the enzyme was passed through a 5 mL HiTrap Desalting column. The column was equilibrated with 25 mL of 66 mM phosphate buffer, pH 8. Samples from the affinity chromatography containing ASNase were applied to the column, and immediately, 66 mM phosphate buffer, pH 8, was applied, collecting the fractions shortly after.

**Conjugation reaction and cosolvent influence.** For the conjugation reaction, 8 mg of $mPEG_{12K}$-SC were dissolved in 100 µL of a 0.1 mM hydrochloric acid solution. In a 1.5 mL microcentrifuge tube, 50 µL of the following solvents were added: acetonitrile, dimethyl sulfoxide (DMSO), ethanol, and methanol. Then, 50 µL of 66 mM phosphate buffer containing the protein and 20 µL of the PEG solution were added. After 20 minutes, the resulting mixtures were analyzed on SDS-PAGE gel electrophoresis.

**SDS-PAGE Electrophoresis.** Two gels were prepared: the stacking gel and the separating gel. The stacking gel consisted of 2.1 mL distilled water, 0.5 mL of 30% monomer solution, 0.38 mL of 1M Tris-base (pH 6.8), 0.03 mL of 10% SDS, 0.03 mL of 10% ammonium persulfate (APS), and 0.003 mL of Temed. The 12% separating gel was composed of: 1.7 mL distilled water, 2.0 mL of 30% monomer solution, 1.3 mL of 1M Tris-base (pH 8.8), 0.05 mL of 10% SDS, 0.05 mL of 10% APS, and 0.002 mL of Temed. For each sample to be analyzed, 15 µL of the sample and 15 µL of the sample application solution (1.25 mL of 1M Tris-base, 2 mL of 10% SDS, 0.5 mL of mercaptoethanol, 4 mL of 0.05% bromophenol blue, 1 mL of glycerol, and 1.25 mL of distilled water) were added to a 250 µL microcentrifuge tube. The samples were heated in a water bath at 95°C for 10 minutes. Then, 30 µL of sample was applied to each well. The gel tank was connected to the power supply and set to a voltage of 130V. When the samples reached the bottom of the gel, the power supply was turned off. The gel was placed in a container, and the staining solution was added, allowing it to contact the gel for about an hour and a half. After this time, the staining solution was removed, and the destaining solution was added, which remained in contact with the gel overnight. Finally, the gel was photographed.

## 3 RESULTS & DISCUSSION

The L-asparaginase production was carried out through autoinduction, and the enzyme obtained after size exclusion chromatography without imidazole was used in the conjugation reaction with PEG. Figure 1 shows the gel electrophoresis obtained for the study of cosolvent influence. Numbers 1, 2, 3, 4, and 5 indicate the conjugated samples using acetonitrile, DMSO, ethanol, methanol, and phosphate buffer, respectively.

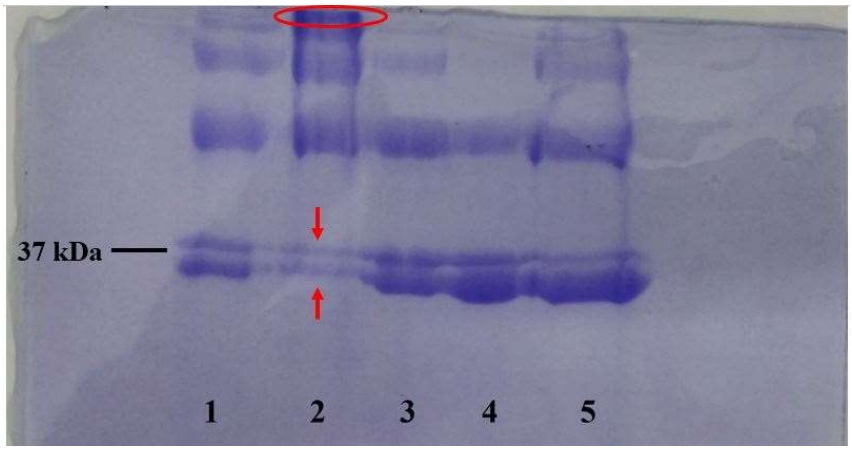


**Figure 1**. 12% polyacrylamide gel with products from the conjugation reaction in different cosolvents. (1) acetonitrile; (2) DMSO; (3) ethanol; (4) methanol; (5) phosphate buffer.

When comparing the bands located in the range of 37 kDa with those situated above this size, the conjugation of recombinant *E. coli* L-asparaginase is confirmed, as expected, since this result has already been obtained by other research works from the research group[16, 17]. $PEG_{12K}$-SC forms a urethane bond with the lysines present in the proteins, and the recombinant ASNase from *Z. mobilis* has 21 lysines in each tetramer unit. However, PEG conjugation to the enzyme will only occur in those lysines that are exposed[16].

As we can observe, the conjugation reaction occurred in the presence of all cosolvents. In the presence of the cosolvents acetonitrile and DMSO, the formed conjugates have bands located in the same size range, suggesting that the stoichiometry is the same. However, a higher conversion into conjugated species was achieved in the presence of DMSO, as evidenced by a greater intensity of bands located above the 37 kDa range. Additionally, the band corresponding to it showed low intensity in the area corresponding to the enzyme that was not conjugated (area between the red arrows).

DMSO was used as a solvent in the conjugation of PEG to Granulocyte Colony-Stimulating Factor (G-CSF). Two polymers were employed for this purpose, PEG-maleimide (MAL-PEG) and PEG-succinimidyl carbonate (PEG-SC). When compared to conjugation in water, it was observed that the degree of conjugation in the organic solvent increased by 33% for MAL-PEG and 42% for SC-PEG. Upon analyzing the protein's conformation in aqueous solution and in the pure solvent using circular dichroism and fluorescence spectra, the authors observed that its structure was unfolded, leading to the conclusion that this exposes the amino acids susceptible to PEG binding more to the solvent, thus facilitating conjugation[18].

The effect of acetonitrile addition was investigated in a biphasic aqueous system containing PEG (at different molecular weights), $K_3PO_4$, and water. The addition of 5% by weight of this solvent resulted in an enhancement of the salting-out effect, favoring the

formation of larger biphasic regions. However, this effect was more pronounced in systems with lower molecular weight polymers, showing no significant effects in systems formed by PEG with a molecular weight greater than 6000 g/mol. The PEG used in the experiments has a molecular weight of 12000 g/mol, so it is plausible to assume that the addition of acetonitrile as a cosolvent did not interfere with a possible salting-out phenomenon, thus not negatively affecting the conjugation of PEG with ASNase[19].

Conjugation in the presence of methanol occurred at lower levels compared to the other cosolvents. The presence of only one band at the top of the gel indicates that perhaps with this solvent, only the conjugate with a single PEG chain per protein monomeric unit is formed.

The influence of DMSO, ethanol, and methanol was evaluated in site-specific conjugation, using the enzyme transglutaminase, of PEG to salmon calcitonin and growth hormone. As a result, researchers obtained only mono-pegylated conjugates[20]. In this work, DMSO and ethanol produced conjugates with different stoichiometries, while methanol showed only one band located in the same size range. However, it is worth noting that the conjugation reactions performed in this work are random, and to determine whether the formed conjugates are mono-, di-, tri-, or poly-pegylated, the application of another analytical technique such as size exclusion chromatography is necessary[21-23].

## 4 CONCLUSION

In this work, the conjugation reaction of PEG to recombinant *Z. mobilis* L-asparaginase was carried out in the presence of the cosolvents acetonitrile, DMSO, ethanol, and methanol. Based on the results obtained, it is evident that to achieve a PEG-ASNase conjugate with the highest number of polymeric chains per enzyme, DMSO yielded the best outcome. However, an interesting observation was the presence of only one band after enzyme conjugation in the presence of methanol. In the literature, there are few reports on the influence of cosolvents on the conjugation reaction, but those that are described show a positive influence on the reaction, depending on the objective to be achieved. Thus, further analyses should be conducted, including those that can identify the degree and yield of the conjugation product.

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