

PEG PEPTIDE AND PROTEIN DRUG DELIVERY: A PROCEDURE TO IDENTIFY THE PEGYLATION SITE

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Introduction

PEGylation is an already established methodology to overcome problems connected with the use of peptides and proteins in therapy namely immunogenicity, proteolytic degradation and rapid clearance from circulation¹.

One of the still open problems connected with this technique is a friendly method for the characterization of the protein PEG conjugates. This is due to the fact that peptides and proteins have several sites of conjugation with different reactivity or accessibility and therefore the PEGylation product is a mixture of isomers that may differ in biological activity.

The analytical characterization of the isomers is a difficult task because the PEG chains, for their great hindrance, make it difficult in most of the cases the separation of conjugates with the available chromatographic techniques. Furthermore the proteolytic digestion of the protein conjugates to obtain peptides of a size suitable to be sequenced may be prevented by the presence of PEG.

These drawbacks are overcome by the procedure presented here, based on the selective removal of the PEG chain that leaves the unnatural amino acid norleucine as suitable reporter group attached to the protein where PEG was linked.

This goal is achieved by the use of the following activated polymer for the conjugation: PEG-Met-Nle-OSu.

Once bound to the protein, the polymeric moiety is removed by CNBr treatment leaving Nle only attached to the protein. The Nle-protein conjugate may thus be easily sequenced as a normal protein and the PEGylated site identified by AAA or mass spectrometry.

Experimental Methods

The polymer PEG-Met-Nle-OSu was prepared by reacting PEG, activated as p-nitrophenylcarbonate¹, with H-Met-Nle-OH in 50%

acetonitrile at pH 8, followed by purification with ionic exchange chromatography and activated with N-hydroxysuccinimid.

Insulin was dissolved in DMSO, the activated PEG-Met-Nle-OSu was added in a ratio of 1 polymer chain per insulin molecule and the product was purified by reversed phase HPLC. The conjugate was isolated, lyophilized, treated with CNBr in 70% formic acid and chromatographed again. The resulted Nle-insulin was reduced with 1,4-dithio-L-threitol and carboxymethylated. The two insulin chains were separated by HPLC and analyzed by amino acid analysis and sequence.

Lysozyme was modified with a 30 molar excess of polymer at pH 8.5 and purified by gel-filtration chromatography with Superose 12TTM. The conjugate was treated with CNBr as above and again fractionated by gel-filtration chromatography.

Results and Discussion

The gel-filtration analysis of native lysozyme, PEG conjugated lysozyme and lysozyme after PEG removal is reported in Figure 1. As expected the PEGylation is accompanied by reduction in elution volume with respect to the native product, whereas following CNBr treatment the product is eluted at an elution volume close to that of the native lysozyme. The small difference in elution is due to the increased volume of the protein for the bound norleucine. Analysis of the last product performed by iodine assay³ or mass spectrometry indicated the complete removal of PEG from the protein while the amino acid analysis after acid hydrolysis showed the presence of norleucine.

Insulin is eluted from the HPLC column at 18.32 minutes whereas PEGylated insulin is eluted at 22.07 minutes and the CNBr dePEGylated form at 18.70 minutes. Again as for lysozyme, the insulin PEGylation is accompanied

by increased volume, reflected in a great difference in elution time, whereas the dePEGylated insulin has an elution volume close to that of the native peptide. The final product was reduced and carboxymethylated to block the SH groups and the two insulin chains were separated. By aminoacid analysis and mass spectrometry it was possible to demonstrate that the reporter Nle was present in the β chain. Finally one step of Edman degradation on the β chain released Phe and Nle indicating that the α -amino phenylalanine was not PEGylated and consequently that PEG was bound to lysine in position 29.

Conclusion

Among the analytical characterizations useful for approval of new PEGylated drugs it is the evaluation of the conjugation site. This is very difficult when PEG is attached to the peptide or protein chain because of its great hindrance. A removal of the polymer leaving on the protein chain a suitable reporter group that can be easily identified, may be a solution.

This approach is well demonstrated in the present research that shows the possibility to remove PEG in mild conditions from lysozyme and insulin and to identify the site of conjugation by the classical procedures of peptide sequence in the case of insulin.

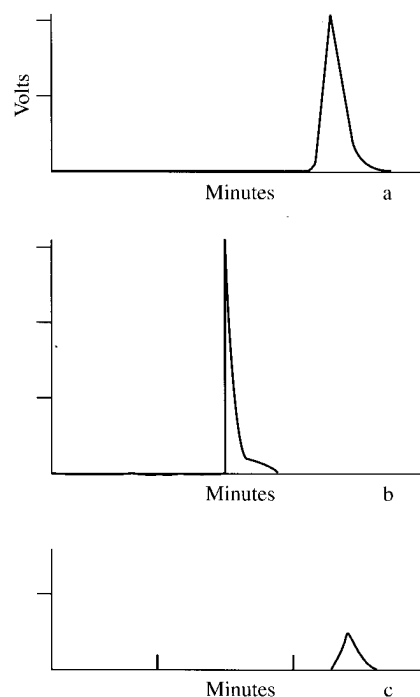


Fig. 1. Chromatographic profile of a) native lysozyme b) PEG-Met-Nle-conjugate lysozyme c) Nle-lysozyme obtained from CNBr cleavage of PEG-Met-Nle-conjugate lysozyme.

References

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