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# One-pot labeling and purification of peptides and proteins with fluorescein maleimide

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**Abstract**—Fluorescein labeling of peptides and proteins is required for numerous biophysical or biological experiments such as fluorescence microscopy, fluorescence resonance energy transfer (FRET) or fluorescence imaging. The commonly used strategy relied on the coupling of the dye reagent followed by a gel filtration to recover the labeled molecule. Here we report a simplified method for the labeling of peptides and proteins on a cysteine residue and their purification. The method is based on the precipitation of peptides and proteins in acetone, fluorescein maleimide being soluble in this solvent. The excess of dye is fully eliminated after a couple of acetone washes and the precipitated peptide or protein is readily recovered. 

Fluorescein labeling of peptides and proteins is usually performed through either the side chain amino group of a lysine residue or the side chain sulhydryl group of a cysteine residue, using commercially available linkers. These include fluorescein-succinimidyl and -maleimide derivatives for specific labeling of $NH_2$ or SH sites respectively. However, a large excess of the fluorescent dye is usually required to achieve a good yield of the labeled compound and therefore an additional step for the removal of this excess of dye reagent is necessary after the labeling reaction. A gel filtration column is usually performed,[7,8] but dialysis or high performance liquid chromatography can also be adopted.[3,4] These purification procedures are time consuming, and, more importantly, they could lead to a significant loss of material by non-specific interactions with the matrix during the gel filtration procedure or by several consecutive handling. This is particularly important with synthetic peptides or with proteins only available in limited quantities.

Our initial intention was to label small quantities (10 μg) of a recombinant GST-Tat fusion protein for cellular microscopy purpose[2] through their cysteine side chains. To avoid the gel filtration step recommended by the supplier after the labeling step and the subsequent loss of material, we investigated an alternative one-pot strategy to retain the fluorescein maleimide in solution while precipitating the protein. Several organic solvents could be used for such strategy. However acetone was selected since this solvent allows a complete extraction of the fluorescein maleimide dye and the full precipitation of peptides and proteins. Moreover acetone precipitation of the labeled peptide or protein was directly performed in the tube where the labeling step was applied. Indeed, this should allow its quantitative recovery after a single centrifugation step.

A 14 amino acids peptide corresponding to the basic domain of the HIV-1 Tat protein (sequence H-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Cys-OH) was first used to validate the method since it was available in quantity in our laboratory[4] and contained a single terminal cysteine residue. Moreover such labeled cell penetrating peptide (CPP) could be useful for studying the cellular uptake in various conditions as previously stated.[4] This peptide was obtained by solid phase peptide synthesis using the Fmoc chemistry on a Pioneer Synthesizer (AppliedBiosystems) as previously described.[6] After deprotection, the Tat peptide was purified to homogeneity and fully characterized by analytical HPLC, amino acid analysis and mass spectrometry (data not shown). The Tat peptide was processed for labeling with fluorescein using a fluorescein 5-maleimide derivative (Molecular Probes). According to the manufacturer protocol, the coupling step was performed in a final volume of 50 μl in 100 mM of Tris buffer pH 6.8 with 10 molar excess of fluorescein

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5-maleimide for 2 h in the dark. Then 500 μl of cold acetone was added to the labeling mixture. The tube was agitated with a Vortex for 15 s and precipitation was allowed for 30 min at −20°C. The tube was then centrifugated at 30,000*g* at 4°C and the supernatant was carefully removed. The peptide pellet was resuspended in 100 μl of Tris buffer (100 mM pH 6.8) and again precipitated with acetone (1 ml) as described above. In order to detect by HPLC the fluorescein maleimide in the different supernatants, acetone was

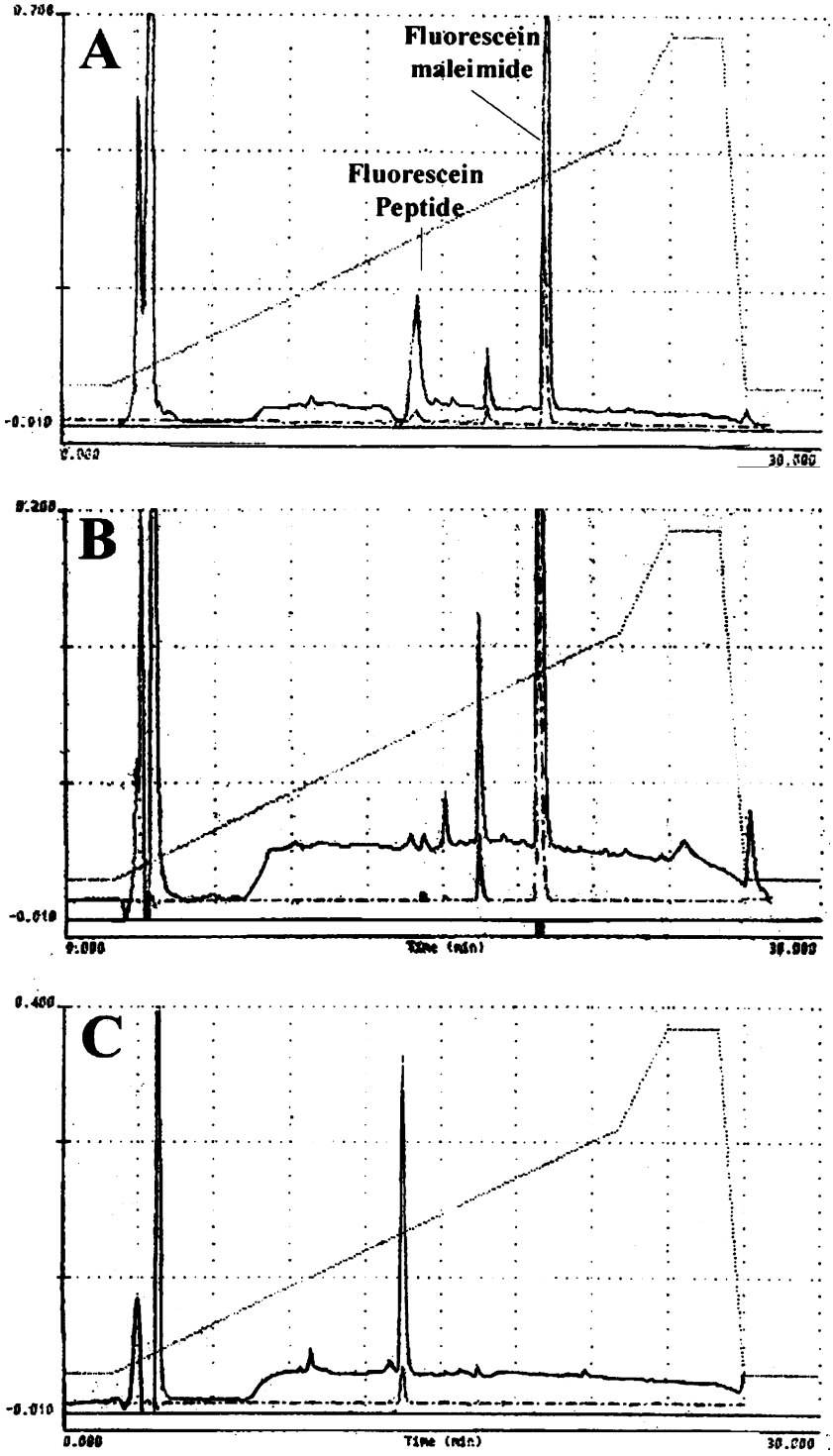


**Figure 1.** HPLC profiles of the different fractions. Chromatography was performed on a C18 Kromasil 5 μm column (250×4.6 mm) using a linear gradient from 10 to 70% of B in 20 min (solvent A; $H_2O$/TFA 0.1%, solvent B: acetonitrile/TFA 0.08%). Wavelength detection was followed at 215 nm (upper profile) and 440 nm (lower profile). (A) reaction mixture at the end of the labeling step. (B) Concentrated fraction of the acetone supernatant after extraction of the fluorescein maleimide. (C) Labeled peptide after resuspension of the acetone precipitated material.

first fully evaporated under nitrogen. Then 100 μl of Tris buffer pH 7.2 was added to the remaining aqueous solvent and the samples were analyzed by HPLC to quantify the efficiency of the extraction procedure. Only a very low amount (<0.1%) of peptide could be detected in the first supernatant fraction since most of the peptide was acetone precipitated (Fig. 1). Moreover, after resuspension of the peptide pellet, HPLC analysis and calculation of the integrated surfaces of the different peaks revealed that more than 98% of the initial fluorescein maleimide was removed in the first supernatant. The additional step of acetone precipitation allowed the complete removal of the remaining fluorescein maleimide from the labeled peptide solution. We thus confirmed the high solubility of the fluorescein maleimide in acetone and the good partitioning of the peptide from the acetone fraction.

Similar experiments were then performed with a longer peptide ($Tat_{20–60}$) also derived from the Tat protein sequence. However, the higher number of cysteine residues in this peptide (7 instead of 1), imposed to adjust the excess of fluorescein maleimide was adjusted to 2 molar excess over cysteine in order to label statistically one or two cysteine residues per molecule of peptide. Despite this change, identical results with complete precipitation of the peptide and full extraction of the excess of fluorescein maleimide were obtained after three acetone precipitation steps. We then labeled a large 27 kDa recombinant protein, namely a GST-Tat fusion protein,[2] using the protocol described above for the shorter $Tat_{20–60}$ peptide. Again, similar data were obtained. It is noteworthy that the fluorescein-labeled recombinant protein was obtained in quantitative yield due to the absence of protein handling between the labeling step and the final resuspension of the protein in the appropriate buffer for further biological experiments. Along this line, this fluorescein labeled GST-Tat fusion protein prepared by this way was shown by fluorescent microscopy to be internalized in cells in agreement with results previously described.[1,5]

Since all the experiments described above were performed with quite similar molecular entities (namely the Tat protein or Tat derived peptides), additional peptides available in our laboratory with a cysteine residue at different position were also subjecteded to this protocol.

These peptides with unrelated sequences (Table 1) were quantitatively fluorescein-labeled and recovered by acetone precipitation from the reactional mixture containing the excess of fluorescein maleimide.

Acetone precipitation of long peptides and more importantly of proteins should lead to their partial or complete denaturation. In most instance however, short peptides are easily renaturated when resuspended in aqueous solution. If not, a renaturation step (as dialysis for instance) could be performed.

**Table 1.** Primary sequence of the peptides labeled in this study (the cysteine of the synthetic peptide is underlined)

| |
|---|
| Recombinant protein : |
| Glutathione S-Transferase-Tat (fusion construct GST-Tat) |
| Synthetic peptides : |
| ACTTCYCKKCCFHCQVCFTTKALGISYGRKKRRQRR RPPQ |
| GRKKRRQRRRPPQC |
| C(Acm)FITKALGISYGRKKRRC |
| EPVDPRLEPWKHPGC |
| CPPLSMPRFMDYWGLNENG |
| WAGGDACGE |

In conclusion, this simplified method for recovering peptides or proteins labeled with fluorescein on a cysteine residue could be a very useful protocol especially to prevent unfortunate loss when small amounts of material are available. However, this labeling technique has also been performed for the labeling of several milligrams of peptides.

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