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Notes & Tips

A simultaneous colorimetric assay of free and protein-coupled polyethylene glycol

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We show in this report that we can accurately estimate free and coupled forms of PEG¹ in a model PEGylated protein by hydrolysis of protein-coupled PEG prior to measurement in a biphasic colorimetric system.

Nag et al. [1] previously developed a colorimetric assay for PEG, which is based on partitioning of a chromophore present in an ammonium ferrothiocyanate reagent from the aqueous to the chloroform phase in the presence in PEG. However, this method is limited to PEG proteins that have the same partition coefficient in the biphasic system as the corresponding free PEG.

PEGylated recombinant methionine α , γ -lyase (rMETase) was used as a model PEGylated protein to develop the new PEG assay. rMETase is derived from *Pseudomonas putida* and is composed of four identical subunits with a molecular weight of 172 kDa [2]. PEG-rMETase, possessing beneficial pharmacological properties, is under development as a broad anticancer agent [3].

rMETase was produced in *Escherichia coli* [2]. Monomethoxy polyethylene glycol succinimidyl glutarate 5000 (MEGC-5000) was a gift from the NOF corporation (Tokyo, Japan). Sephacryl S-200 and DEAE-Sephacryl FF were purchased from Pharmacia (Uppsala, Sweden). Fluorescamine and ammonium thiocyanate were obtained from Fisher Scientific (Fairlawn, NJ). Anhydrous ferric chloride was obtained from Aldrich Chemical (Milwaukee, WI). Centriprep YM-30 was purchased

from Millipore (Marlborough, MA). All chemicals used were of analytical reagent grade.

To synthesize PEG-rMETase, MEGC-PEG was used at a molar ratio of MEGC-PEG to rMETase of 45:1. PEG-rMETase was purified on a Sephacryl-200 HR column (HiPrep 26/60, Pharmacia) in order to remove free PEG. The fractions containing rMETase activity were pooled and further purified on a DEAE-Sephacryl FF column (XK 16/15, Pharmacia) to remove free rMETase and partially modified rMETase. The collected fractions were combined and concentrated with a Centriprep YM-30. The protein content of PEG-rMETase was determined by the Lowry method using bovine serum albumin as the standard. PEG-rMETase was compared with native rMETase on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels.

To determine the extent of PEGylation directly, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed with a PerSeptive Biosystems Voyage-Elite mass spectrometer with delayed extraction. The extent of PEG modification of rMETase was indirectly assayed by estimating free amino groups available in the PEG-conjugated enzyme compared to that in the native enzyme using the fluorescamine method [4,5].

For measurement of coupled PEG in PEG-rMETase in the biphasic colorimetric assay, the PEG-rMETase was first subjected to alkaline hydrolysis in 5 N NaOH and then measured in the biphasic colorimetric assay. The test sample (800 μ l) consisting of PEG-rMETase (10 mg/ml) was mixed well with sodium hydroxide solution (5 N, 16 μ l) at room temperature. To ascertain the time to completely release PEG from PEG-rMETase, aliquots (100 μ l) were withdrawn from the above mixture at various time points followed by neutralization with 5 N hydrochloric acid. The extent of hydrolysis

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¹ Abbreviations used: PEG, polyethylene glycol; rMETase, recombinant methionine α , γ -lyase; MEGC, monomethoxy polyethylene glycol succinimidyl glutarate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization.

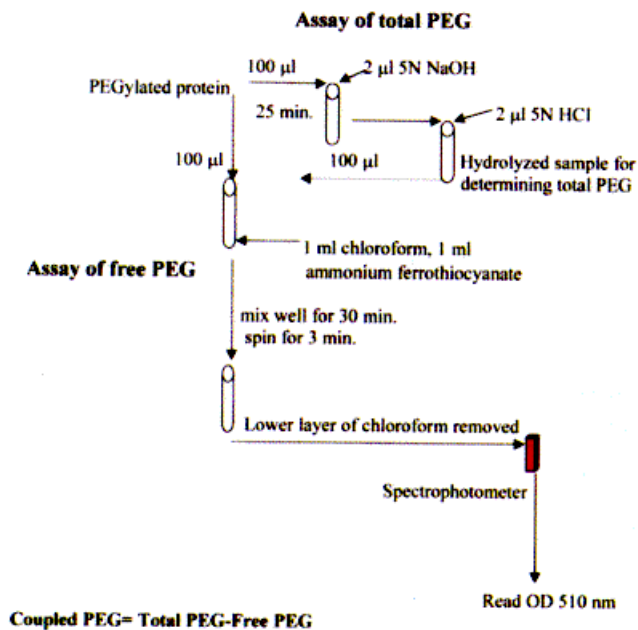


Fig. 1. Colorimetric assay of couple PEG in PEGylated proteins.

was time dependent as determined by SDS-PAGE. One milligram of hydrolyzed PEG-rMETase in 100 µl sodium phosphate buffer (pH 7.4) was added to the biphasic assay system comprising 1 ml chloroform and 1 ml aqueous ammonium thiocyanate (0.1 M) (Fig. 1). After vigorous vortexing for 30 min and centrifugation at 3500g for 3 min, the two separated phases were collected. The aqueous phase, containing precipitated rMETase, was centrifuged at 3000g for 2 min. The pellet was suspended in 20 mM sodium chloride solution and then centrifuged at 3000g for 2 min. The precipitate was dissolved in Tris-glycine SDS sample buffer and analyzed by 10% SDS-PAGE. The chloroform phase was

evaporated under reduced pressure. The residue was reconstituted by introducing 20 µl of sodium chloride (20 mM) solution. Protein content present in the solution was analyzed by 10% SDS-PAGE and the Lowry method. The amount of coupled PEG in PEG-rMETase can be determined from the total PEG content after complete hydrolysis minus the PEG content measured before hydrolysis (Fig. 1).

The conjugated protein showed a diffuse band of high molecular weight on SDS gels due to varying degrees of PEGylation in the rMETase population and polydispersity of PEG. No discernible band corresponding to unconjugated rMETase was found with PEG-rMETase, indicating modification of all subunits of the parent protein. A small amount of free PEG was present in the purified PEG-rMETase even though several steps of purification had been carried out. These results indicate that it is necessary to quantify free PEG as well as coupled PEG in PEG-rMETase preparations.

MALDI mass spectrometry results indicated PEG-rMETase consisted of 5 peaks corresponding to molecular weight 5126 (free PEG); 58901 (3 PEGs coupled per subunit); 64637 (4 PEGs coupled to each subunit); 69811 (5 PEGs coupled to each subunit); and 75110 (6 PEGs coupled to each subunit). No unmodified rMETase peak appeared. These results further demonstrated a small amount of free PEG in the final product. The results also demonstrated that free PEG should be subtracted from the total PEG content detected in PEG-rMETase in order to obtain accurate results on the extent of modification of PEG proteins.

After vigorously mixing the biphasic system with 1 mg of unhydrolyzed PEG-rMETase for 30 min, it was observed that PEG-rMETase precipitated in the aqueous phase. These data indicate that the residual free PEG in purified PEG-rMETase can be effectively ex-

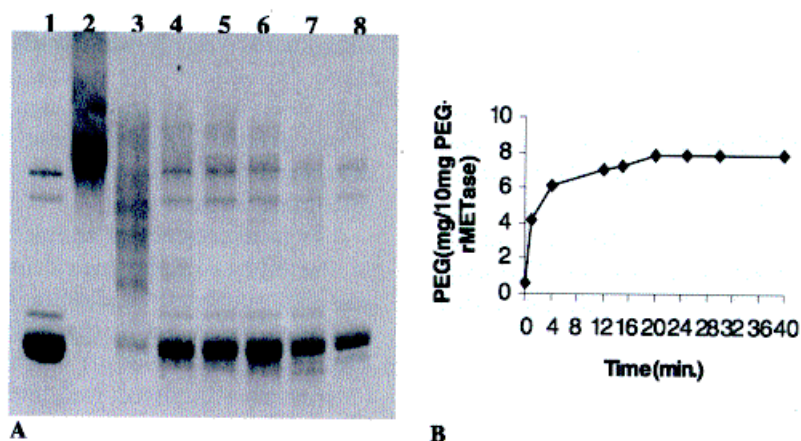


Fig. 2. Kinetics of release of PEG from PEG-rMETase by alkaline hydrolysis. (A) PEG-rMETase analyzed on 10% SDS gels after different times of hydrolysis. (Lane 1, native rMETase; Lane 2, PEG-rMETase; Lanes 3, 4, 5, 6, 7, and 8 correspond to 1, 4, 12, 15, 20, and 25 min of hydrolysis, respectively). (B) PEG released from PEG-rMETase by alkaline hydrolysis at varying time points measured in the biphasic colorimetric assay. See Materials and methods for details.

tracted by the chloroform phase. However, PEG-rMETase does not partition in the chloroform of the two-phase system. The data suggest that the biphasic assay system is not suitable for directly measuring PEG in PEG-rMETase, because it cannot extract PEG-rMETase into the chloroform phase.

However, PEG can be released rapidly from the PEG-rMETase conjugate under strong alkaline conditions and then be assayed in the biphasic colorimetric system. Fig. 2 shows the kinetics of hydrolysis of PEG from the PEG-rMETase. Both SDS-PAGE (Fig. 2A) and the PEG-release curve (Fig. 2B) indicate that it took approximately 25 min to completely release coupled PEG from PEG-rMETase. When the hydrolysis reaction time was prolonged, the amount of detectable PEG did not increase. Therefore, 25 min was taken as the time to hydrolyze PEG-rMETase for measurement in the biphasic colorimetric assay.

To validate the strategy proposed in this report, several batches of PEG-rMETase were assayed for PEG content by both the biphasic colorimetric assay combined with alkaline hydrolysis and the fluorescamine method. The degree of modification of rMETase was calculated based on an rMETase molecular weight 172 kDa containing 36 primary amino groups. The results from the two methods were basically consistent. The colorimetric assay combined with alkaline hydrolysis, however, enables free PEG as well as protein-

conjugated PEG to be quantified. The new method should be useful with numerous PEG proteins to monitor PEGylation reactions and stability.

Acknowledgment

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