Analysis of Sulfur-Containing Amino Acids

III. Alkylation of Cysteine

The free sulfhydryl group of cysteine (Cys) readily reacts with a number of alkylating reagents, and the reactions form the basis of many popular techniques for Cys quantitation. Benefits to alkylation include greater stability during acid hydrolysis, single product of derivatization with PITC, and favorable reaction of the sulfhydryl group in comparison to other potentially reactive sites in peptides and proteins.

The most popular reagents for alkylation are iodoacetic acid and iodoacetamide, the products of alkylation being carboxymethylcysteine (CMC) and carboxamidomethylcysteine. The latter is converted to CMC during hydrolysis, and consequently derivatization and analysis of the two is identical. Unwanted sideproducts can arise from reaction with His and Met, although these can be controlled. Another reagent, 4-vinylpyridine is gaining in popularity. The derivative pyridylethyl cysteine (PEC) is more stable than CMC during hydrolysis, and unlike CMC, there are no significant side reactions.

It is important to remember that alkylation reagents do not react with the disulfide link, but with the sulfhydryl group. Thus, for the determination of total sulfhydryl content (Cys plus Cys₂) conversion of Cys₂ to Cys must be carried out prior to alkylation. Common reagents employed for disulfide reduction are mercaptoethanol and dithiothreitol (DTT). The sample is incubated with a reducing agent, in excess of the estimated sample sulfhydryl content, followed by the addition of alkylation reagent in excess of the total of reducing agent plus reactive sulfhydryl.

The typical procedure shown below is taken from the Pico-Tag® Methods Book¹.

Reagents: Guanidinium HCl (Gu-HCl) Electrophoresis grade or better
Dithiothreitol (DTT) Sigma Chemical Co.
4-vinylpyridine (4-VP) Aldrich Chemical Co.

Buffer: 0.5 M N-ethylmorpholinium acetate (Aldrich Chemical Co.), pH 8.3

1. Add water to 6.4 mL of N-ethylmorpholine to bring volume to 100 mL.
2. Titrate to pH 8.3 with acetic acid.

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**Procedure:** The following example can be used for 1-1000 nmol of protein or peptide.

1. Place sample in sealable vial, dry via vacuum, N₂ or lyophilization.
2. Dissolve sample in 1 mL buffer.
3. Add 1g Gu-HCl. Mix
4. Add 4 mg DTT. Mix
5. Blanket sample with N₂, seal tightly, and incubate at room temperature for 4hr.
6. Add 8 μl 4-VP, blanket with N₂, seal, and incubate at room temperature for 4-16hr.
7. Add 3 mL water
8. Desalt by HPLC or dialysis.

To limit reaction volume, reduce buffer to 0.25 mL, Gu-HCl to 250 mg, DTT to 1 mg and 4-VP to 2 μl. This will be adequate for 0.25-250 pmol of sample. After alkylation dilute with 750 μl H₂O and proceed.

**Calibration Standard**
- Make 2.5 mM solution of pyridylethyl cystine (PEC) in 0.1M HCl
- Add 200 μl hydrolyzate standard (Waters P/N 88122) to 200 μl PEC solution (PEC calibration mixture).
- Derivatize 10 μl, inject 4 μl to calibrate at the 250 pmol level

**Note:**
- 8 μl 4-VP is approximately 74 μmol. Substitution of other alkylation reagents for 4-VP (e.g., iodoacetic acid) in the procedure should use the same reagent concentration.

Separation of CMC or PEC-containing samples is accomplished using the standard hydrolysate chromatographic conditions (Figure 1) as described in the Pico-Tag Operators Manual and LAH #0363.

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