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Attachment of a single fluorescent label to peptides for determination by capillary zone electrophoresis

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ABSTRACT

Complicated electropherograms are produced in the separation of fluorescently labeled peptides. Incomplete labeling of ε-amino groups on lysine residues results in the production of $2^n - 1$ reaction products, where n is the number of α and ε amino groups in the peptide. A single label is attached to the peptide by first taking the peptide through one cycle of the Edman degradation reaction. All ε-amino groups are converted to the phenyl thiocarbamyl and the cleavage step exposes one α-amino group at the N-terminus of the peptide; the fluorescent label is attached to the N-terminus.

INTRODUCTION

Capillary zone electrophoresis is a particularly powerful technique for the separation and analysis of complex protein mixtures [1]. To avoid column overloading, only small amounts of dilute sample can be introduced onto the capillary without degrading the separation efficiency. For example, injection of 1 nl of a 10^{-6} M protein solution corresponds to the introduction of 1 fmol of protein. Conventional ultraviolet absorbance detection is problematic at low femtomole sample loading. Universal detection, for example based on refractive index gradient detection, is useful in the analysis of unlabeled peptides [2]. Again, detection limits are in the low femtomole range. Alternatively, postcolumn derivatization may be used to label proteins for fluorescent detection, albeit with limited sensitivity and degraded separation efficiency [3,4].

Fluorescence detection provides outstanding detection limits for capillary electrophoresis. Labeled amino acids and DNA sequencing fragments may be detected at the low to sub-zeptomole level [5]. While precolumn fluorescent labeling is useful in amino acid and DNA analysis, it is not always useful in peptide analysis. The difficulty is straightforward: protein labeling reactions inevitably rely on reagents that attack the n-terminal α-amino group. Simultaneously, the ε-amino group associated with lysine residues will also react. Unfortunately, most fluorescent labels are bulky and labeling does not go to completion. The labeling reaction produces a complex mixture of products, corresponding to attachment of different numbers of labels at different sites [6]. Each of the reaction products has slightly different migration rate, giving rise to a complicated and uninterpretable electropherogram.

The number of possible reaction products may be calculated from simple combinatorial analysis. If there is no lysine residue, there is only one possible reaction product, that labeled at the N-terminal amino group. If there is one lysine group, there are three possible labeled products: one labeled only at the N-terminal α-amino group, one labeled only at the lysine ε-amino, and one with both sites labeled. If there are n primary amino groups that can be labeled, then there are

$$\sum_{m=1}^{n} \frac{(n)!}{m!(n-m)!} = 2^n - 1$$

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different labeled products possible. For peptides and proteins with more than a few primary amino groups, the number of reaction products becomes very large. A peptide with three primary amino groups has seven possible labeled reaction products while a protein with 10 primary amino groups has 1023 possible products. Analysis of a mixture of peptides that are incompletely labeled is not practical; instead of observing a single peak for each peptide, many tens or hundreds of peaks are observed for each peptide and protein.

To ensure that only the N-terminus amino group is labeled, the peptide may be taken through one cycle of the Edman degradation reaction before the labeling reaction. This method first treats the sample with phenyl isothiocyanate (PITC), the classic Edman degradation reagent [7]. PITC is relatively small and efficiently reacts under basic conditions with all primary and secondary amines present in the protein. Under acidic conditions, the N-terminal amino acid is cleaved, exposing an unreacted primary amine. This peptide, now truncated by one residue, may be labeled with an appropriate fluorescent (or chromophoric) reagent. We chose to use fluorescein isothiocyanate as the labeling reagent, but other isothiocyanates or sulphonyl halides could be used. The product of the reaction is the peptide with the original N-terminal amino acid removed, with all ε-amino lysine groups converted to the phenyl thiocarbamyl, and with a single fluorescent label at the N-terminus of the truncated peptide.

EXPERIMENTAL

Peptide 8656 (Peninsula Labs., Belmont, CA, USA) has a primary structure Arg–Lys–Arg–Ala–Arg–Lys–Glu. A 10^{-4} *M* solution was prepared in a 0.2 *M* pH 9.2 borate buffer. The fluorescein derivative was prepared by mixing 100 μl of $4.7 \cdot 10^{-4}$ *M* fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, OR, USA) solution prepared in acetone with 100 μl of the peptide solution. The reaction proceeded at room temperature in the dark for 8 h.

The manual Edman degradation procedure was similar to that of Edman and Henschen [8]. Approximately 2 mg of the peptide were dissolved in 1 ml buffer in a stoppered glass test tube; the buffer was 0.4 *M* triethylamine (Anachemica, Montreal, Canada) in 1-propanol–water (3:2. v/v), adjusted to pH 9.6 with 1 *M* trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA). A 50-μl volume of PITC (Sigma) was added to the peptide solution, the tube was flushed with nitrogen and incubated at 55°C for 20 min in a water bath with occasional agitation. The solution was extracted with five 2-ml aliquots of benzene with centrifugation to separate the phases. The benzene layers were discarded and the aqueous layer was freeze dried. The residue was extracted three times with 0.5-ml aliquots of ethyl acetate (Caledon Labs., Georgetown, Canada). The ethyl acetate had been passed through an alumina column and filtered before use. The ethyl acetate extract was dried under a stream of nitrogen. A 100-μl volume of anhydrous TFA (Protein Sequencing Grade, Sigma) was added and the solution was incubated at 40°C for 15 min in a water bath with occasional agitation. The residue was dried under vacuum for about 10 min. A 3-ml volume of 1,2-dichloroethane (Caledon Labs.) was added and the solution was centrifuged to separate the layers. The dichloroethane layer was discarded, and 1 ml more dichloroethane was added. The residue was macerated with a glass stirring rod. The mixture was centrifuged and the dichloroethane layer was discarded. The aqueous layer was dried under vacuum and stored in a desiccator at 4°C until further use.

The dried, truncated peptide was dissolved in 1.00 ml of a 0.2 *M* pH 9.2 borate buffer, with concentration *ca.* 10^{-5} *M*. To label the truncated peptide, 10 μl of $4.7 \cdot 10^{-4}$ *M* FITC solution prepared in acetone was mixed with 100 μl of the truncated peptide solution. The reaction proceeded for 8 h in the dark at room temperature. Solutions were diluted to 10^{-6} *M* with 5 m*M* borate buffer before injection.

The capillary electrophoresis system was identical to that described before [9]. A 44 cm × 50 μm I.D. capillary was used for the separation. The separation buffer was 5 m*M* pH 9.2 borate buffer. Samples were injected for 5 s at 2 kV; separation proceeded at 13 kV.

RESULTS AND DISCUSSION

The primary structure of this peptide is Arg–Lys–Arg–Ala–Arg–Lys–Glu. The peptide used in this example has two lysine residues in addition to the N-terminal arginine residue. When labeled with

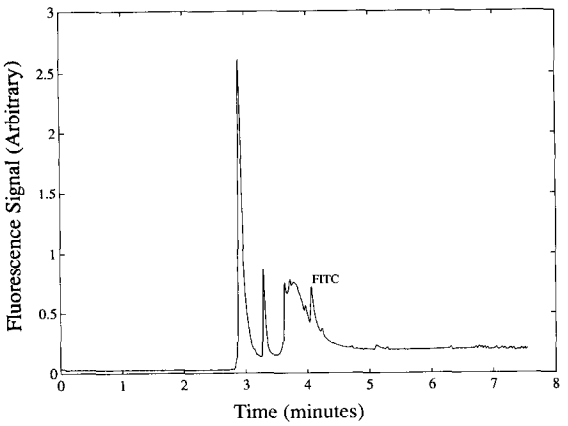

Fig. 1. Capillary zone electrophoresis separation of fluorescein thiocarbamyl derivative of peptide 8656. Peak labeled FITC is the unreacted derivatization reagent.

FITC, a total of seven fluorescent products are possible. The separation of the labeled peptide is shown in Fig. 1. In addition to the unreacted FITC peak, there are at least seven peaks present, corresponding to the different products of the labeling reaction. Comparison with the electropherogram for the single label peptide suggests that the second peak in this electropherogram contains a single label at the α-amino group. The relatively close spacing of the other peaks is not surprising. Conversion of the

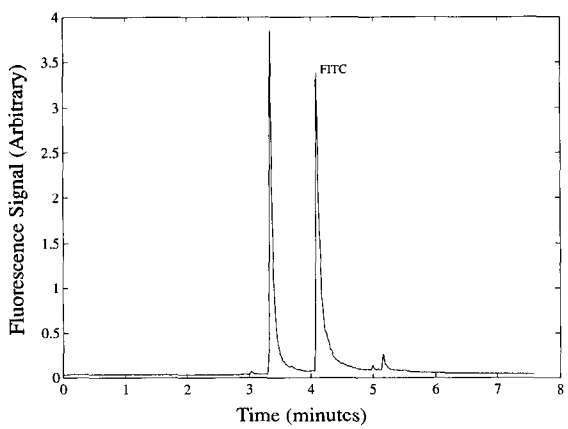

Fig. 2. Capillary zone electrophoresis separation of fluorescein thiocarbamyl derivative of treated peptide 8656. The peptide had been treated with one cycle of a manual Edman degradation; the peptide is truncated by one residue compared with Fig. 1. Peak labeled FITC is the unreacted derivatization reagent.

ε-amino to the phenyl thiocarbamyl derivative increases the size of the peptide slightly but should not change its charge; each positively charged thiocarbamyl replaces a positively charged ε-amino group.

If the peptide is taken through one cycle of the Edman degradation reaction, the N-terminal amino acid is removed; the primary structure of the truncated peptide is Lys–Arg–Ala–Arg–Lys–Glu. In the Edman degradation step, the amino-containing side chains of the lysine residues are converted to the ε-phenyl thiocarbamyl derivative. By protecting the ε-amino group and cleaving the N-terminal amino acid, only one amine is available for further labeling. The reaction product shows two peaks (Fig. 2), one corresponding to the labeled peptide and the other to unreacted fluorescein isothiocyanate. The Edman treatment appears to be quite efficient; no peaks corresponding to multiple labeling are detected.

A small impurity is noted in the reaction product, with an elution time of about 5.25 min. This impurity was undetectable for the labeled native peptide because of the complex reaction mixture. However, by ensuring that only one label is attached to the peptide, the electropherogram is simplified so that the minor contaminant can be detected.

CONCLUSION

Precolumn fluorescent labeling is convenient for high-sensitivity peptide analysis in capillary electrophoresis. When precolumn derivatization is employed, incomplete multiple labeling is problematic, producing a complicated mixture from a single analyte. A single label can be attached to the peptide by first taking the peptide through one cycle in the Edman degradation reaction; because of its small size and high reactivity, phenyl isothiocyanate quickly and efficiently labels all free amino groups in the peptide. All ε-amino groups on lysine residues are converted to the ε-phenyl thiocarbamyl, and cannot partake in the labeling reaction; only the N-terminal amino group is available for further labeling.

As a limitation to this technology, only unblocked peptides can be labeled. N-Terminal acetylated peptides do not have a free α-amino group. While various chemical unblocking procedures, such as cyanogen bromide cleavage, can be employed, they

can produce a number of reaction products, defeating the purpose of this labeling protocol. Also, the procedure removes the N-terminal amino acid; two peptides that differ only at the N-terminal amino acid will be indistinguishable after this labeling procedure.

This reaction was performed by use of the manual Edman reaction at relatively high concentration. It would be much more efficient to use an automated protein sequencer to perform this reaction; picomoles of protein could be prepared at 10^{-8} *M* concentration for fluorescent labeling [10]. However, the sequencer must be modified to allow facile recovery of the treated peptide; a strong solvent could be used to remove the peptide from a Polybrene treated solid-phase sequencing disk.

Last, this reaction was demonstrated with PITC as the protecting reagent and fluorescein isothiocyanate as the labeling reagent. It may prove possible to use smaller protecting reagents, such as methyl isothiocyanate, which should react more quickly than PITC. Other fluorescent reagents could be used. As always, the labeling chemistry should be chosen to provide a good match to the excitation source [11]. As an attractive reagent, the 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde derivative absorbs strongly in the blue and appears to react quickly with primary amines [6].

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