PROTEIN EXPRESSION ANALYSIS BY SIGNATURE PEPTIDES USING MICROFLUIDIC 2D SEPARATION DEVICES

R. S. Foote, J. D. Ramsey, S. C. Jacobson, R. S. Ramsey and J. M. Ramsey
Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-6142 USA

ABSTRACT

We are developing methods for analyzing complex protein mixtures via the use of signature peptides that can be rapidly separated using microfluidic devices. Computer analysis of human and *E. coli* protein databases has shown that a high percentage of proteins have unique C- and N-terminal peptide sequences, that can potentially be used for unambiguous identification and quantitation of the parent proteins. A method of isolating N-terminal peptides for this purpose has been demonstrated.

Keywords: protein expression, signature peptides, 2D separation

1. INTRODUCTION

Significant progress has been made in the development of microfabricated devices capable of performing two-dimensional tandem separations of peptides [1-3]. Peak capacities of ~4,500 have recently been achieved by combining a chromatographic (MEKC) separation in the 1st dimension with a fast electrophoretic separation in the 2nd dimension [3]. This method has allowed rapid and highly efficient separations of tryptic peptides from digests of single proteins (Fig. 1). In theory, this approach could be used to analyze complex mixtures of proteins by separating and quantifying peptides in the total protein digest with greater speed and less sample than required for conventional 2D PAGE analysis, and would allow direct interfacing with mass spectrometry. However, even simple organisms contain several hundred to a few thousand expressed proteins and their digests will contain many times that number of peptides. In order to reduce the complexity of such mixtures we are developing methods for isolating N- or C-terminal peptides for use as protein signatures. Each protein chain would then be represented by a

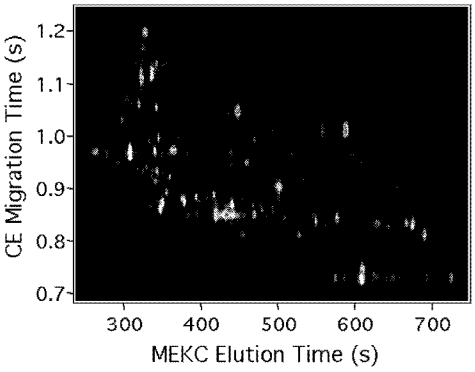

Figure 1. Microchip 2D separation of a human hemoglobin tryptic digest labeled with tetramethylrhodamine.

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single peptide. Computer analysis of *E. coli* protein sequences indicates that approximately 88% of N-terminal peptides and 93% of C-terminal peptides produced by cleavage at arginine residues alone will have a unique amino acid sequence and could therefore provide an unambiguous signature of the parent protein.

2. EXPERIMENTAL

The isolation of N-terminal peptides was demonstrated using lysozyme as a model protein. The protein sample (6.0 mg) was first treated by standard methods to 1) reduce cystine disulfide bonds with dithiothreitol, 2) block thiol groups with iodoacetamide, and 3) block the N-terminal and ε-lysyl amines by reaction with succinic anhydride [4]. Excess reagents were removed by spin filtration and an aliquot (~1 mg) of the modified protein was dissolved in 50 mM sodium bicarbonate containing 0.1% RapiGest SF (Waters) for digestion with trypsin (5 μg/ml, 3h at 37°C). The digest was treated with sulfo-NHS-biotin (Pierce) to biotinylate all newly formed peptide amine termini. The peptides were separated from excess biotin reagent by cation exchange chromatography using stepwise elution with triethylammonium acetate (TEAA) buffer and then subjected to streptavidin chromatography to remove biotinylated peptides from the succinyl-blocked N-terminal fragment. Peptide fractions were analyzed by C18 reverse-phase HPLC using a 0-60% v/v linear gradient of acetonitrile in water, with 0.1% trifluoroacetic acid in both solvents; absorbance was monitored at 214 nm. The isolated N-terminal peptide in 100mM TEAA buffer was analyzed by electrospray ionization time-of-flight mass spectrometry (MS).

3. RESULTS AND DISCUSSION

Lysine-blocked lysozyme contains 11 arginine residues at which trypsin cleavage can potentially occur. The HPLC separation of the resulting peptides is shown in Figure 2 (top trace). Biotinylation of the peptides containing unblocked N-termini altered their elution positions (middle trace). One peptide peak (indicated by arrow) was not shifted by the biotin reaction and was assumed to be the succinyl-blocked N-terminal peptide. This fragment was recovered essentially pure following removal of the biotin-peptides by streptavidin (bottom trace). The overall recovery yield from the initial digest was approximately 90%, based on the HPLC analyses. The identity of the isolated fragment as the N-terminal peptide was supported by its MS analysis. The most abundant sample ion, observed at *m/z* 805.4, corresponds to the expected derivative of the lysozyme N-terminal peptide, KVFGR, in which both the α- and ε-amines on lysine (K) are blocked with succinyl groups (total monoisotopic MW = 805.39). A low intensity ion at *m/z* 1194.5 is believed to be due to the N-terminal peptide of a protein contaminant in the commercial lysozyme sample.

We are presently attempting to apply this experimental method to model protein mixtures for analysis by 2D microchip separation. The signature peptides will be labeled fluorescently for detection and quantitation. A similar chemical approach may be used for the isolation of C-terminal peptides Analysis of both N- and C-terminal peptides will

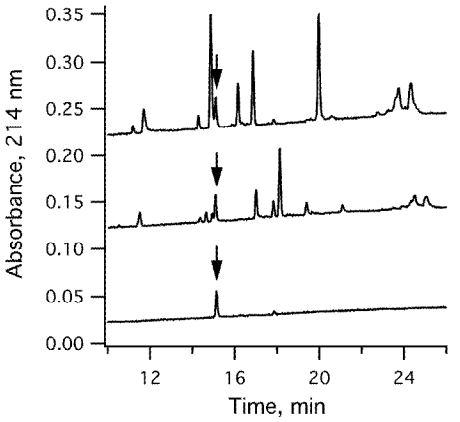

Figure 2. HPLC peptide analyses; see text for explanation.

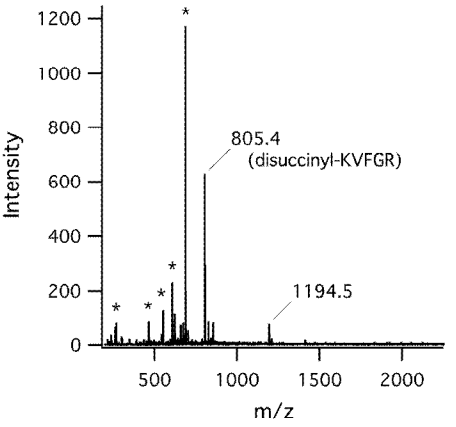

Figure 3. Mass spectrum of N-terminal peptide. *Ions associated with buffer.

provide a high probability (~98%) of observing at least one unique signature for each member of the *E. coli* proteome. These signature peptide approaches may facilitate the analysis of complex protein mixtures by mass spectrometry and allow rapid analysis of protein expression in small numbers of cells using 2D microfluidic separation devices.

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