Capillary Chromatography: Extreme Resolution and Sensitivity in Proteomics LC/MS

LC/MS/MS is a very powerful analytical technique. It can even dissect and analyze regions of chromatograms with overlapping peaks, common when separating complex samples such as enzymatic digests. Figure 1 shows the separation of a tryptic digest of bovine serum albumin (BSA) with MS detection. The amplitude of the trace in Figure 1a is the total ion count (TIC) measured by TOF MS. The chromatogram contains many peaks, quite a few of which are overlapping.

Using the data processing capabilities of the API QSTAR instrument, significant additional information can be derived.

Continued on next page
Figure 1b shows the base-peak chromatogram (BPC) corresponding to Figure 1a. The base peak is defined as the single mass peak with maximum amplitude at each time in the chromatogram. The BPC emphasizes peaks containing a single predominant molecular species and deemphasizes heterogeneous peaks and noise.

Another technique – total ion count based on dependent MS/MS data – is shown in Figure 2. Chromatogram 2a is identical to the chromatogram of Figure 1a. The instrument counts all ions from the TOF mass spectrometer with no ion selection by the quadrupole. In the dependent-data traces, the first quadrupole selects and passes only the most abundant single ion (Fig. 2b) or the second most abundant single ion (Fig. 2c) into the collision cell, just a few seconds each. The TOF mass spectrometer then produces fragment spectra suitable for peptide sequence determination (Figs. 3 and 4). The total ion counts from the TOF spectrum are displayed as amplitude in Figure 2b and 2c. Peptide peaks only partially resolved by the HPLC column are easily revealed by detection dependent on the second most abundant ion (Fig. 2c).

In essence the reversed-phase column and the quadrupole act as orthogonal separation mechanisms to produce pure peptide molecular ions for fragmentation. Complete chromatographic resolution of all peptides is not required. However, the resolution of the chromatographic column simplifies the task of the MS/MS system in selecting ions for sequencing, and the low-TFA capability of the
Practical Application

In a poster presented at the meeting of the American Society for Mass Spectrometry in Chicago, May 27-31, 2001, scientists from the Protein Characterization and Proteomics Laboratory at University of Cincinnati College of Medicine reported using a VYDAC reversed-phase capillary column (Cat. No. 214MS5.310, C4, 300 µm i.d. x 100 mm) together with an Eldex Micropro HPLC system and an Applied Biosystems/MDS SCIEX API 3000™ triple-quadrupole mass spectrometer for detection and identification of expressed sequence tags to identify gene products in *Pseudomonas aeruginosa*. One objective of this work has been to identify proteins that are up-regulated and perhaps essential for anaerobic growth. Such proteins would be potential therapeutic targets for mediation of *P. aeruginosa* biofilms that do not respond to conventional antibiotic therapy and are severely problematic in a number of human diseases including cystic fibrosis.

Proteins from *P. aeruginosa* cultures grown aerobically and anaerobically were separated by 1-D electrophoresis on polyacrylamide gels (Fig. 6). Bands of interest were reduced, alkylated, and then digested in the gel with trypsin. Extracted peptides were applied to the C4 capillary column for separation and analysis. A sample chromatogram is shown in Figure 7, with intensities corresponding to the total ion count (TIC) from the third quadrupole (Q3). Examples of the corresponding mass spectra are shown in Figure 8.

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The API 3000 LC/MS system equipped with Analyst® 1.1 software was used in information-dependent-acquisition (IDA) mode, cycling repeatedly through two experiments: a two-second survey scan during which all ions are passed to Q3 to automatically identify the most intense peptide ion in the 500-1500 m/z range (Figs. 7a and 8a), followed by a four-second MS dependent product-ion scan during which the precursor ion thus identified is selected by the first quadrupole (Q1), fragmented in the collision cell (Q2), and the mass spectrum of the fragments displayed by the Q3 scan (Figs 7b and 8b).

To arrive at peptide sequences and gene assignments, database search software (Proteometrics Sonar MS/MS database search engine) was used to compare the expressed-sequence-tag mass spectra obtained from these experiments to predicted mass spectra using a custom database constructed from DNA sequences for the 5,570 open reading frames encoded in the \textit{P. aeruginosa} genome. Sensitivity using the VYDAC 300 µm i.d. x 100 mm column was estimated from experiments using a tryptic digest of BSA and [Glu1]-Fibrinopeptide B as samples and query to a current NCBI non-redundant database. Reliable sequence assignments were possible with as little as 40 pmole of BSA before digestion and 6.25 pmole of a standard peptide.

Data in this article courtesy of

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Prep 2002
Washington, DC June 16-19, 2002
Paper oxidative stress upon reperfusion following cardiac ischemia is of significant concern in relation to heart attack survival. Myoglobin, a 17-kDa heme protein which normally stores oxygen in muscle tissues has been implicated in lipid peroxidation and protein oxidation during reperfusion. Angelo Filosa, working with Ann M. English at Concordia University, reports using two VYDAC columns in LC/MS applications to investigate oxidation sites in myoglobin (Mb).

Reaction of metMb with H$_2$O$_2$ forms both an oxyferryl heme and an unstable protein-based radical, P•. The specific location of the radical on Mb has been the subject of earlier studies using the spin trap 2-methyl-2-nitrosopropane (MNP).

In this experiment, Mb was incubated with H$_2$O$_2$ at various pHs in the presence of a different spin trap, 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS), then further reacted with ascorbate to reduce spin adducts to more stable spin-paired species. The resulting modified Mb was purified on a 4.6 mm diameter VYDAC column (Fig. 9).

Post-column splitting directed 40 µL per minute of the column effluent to the ESI source of a Finnigan SSQ7000 single-quadrupole mass spectrometer for molecular weight determination. Deconvoluted mass spectra (Fig. 10) show the effects of H$_2$O$_2$ concentration and pH on the products obtained.

**Effects of Oxidation Conditions**

Following digestion with trypsin, tryptic peptides were separated on a 1.0 mm diameter VYDAC column with LC/MS/MS detection using a Waters Alliance/Micromass Quattro spectrometer in positive ion mode between 80-2000 m/z. Peptide mass mapping revealed modified peptides not observed in the native Mb digest (Fig. 11), suggesting radical formation on Tyr103 and Lys42. Sequencing by ESI MS/MS (Fig. 12) confirmed Tyr103 as one site of radical formation.

**Analysis on VYDAC 218TP51**

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**Figure 11.** Analytical separation of tryptic peptides by microbore HPLC. Column: VYDAC 218TP51 5 µm, 300 Å, C18 (1.0 mm i.d. x 250 mm). Flow: 40 µL/min. Mobile phase: A = 5:95 ACN:water, 0.05% TFA. B = 55:45 ACN:water, 0.05% TFA. Gradient: Linear, 0% to 100% B over 20 minutes. Detection: LC/MS/MS. (a) Native Mb. (b) Reduced-DBNBS-Mb tryptic peptide. R-DBNBS-modified peptides 97-118, 103-118, and 42-56 are highlighted (*).

**Figure 12.** LC/MS/MS sequencing of R-DBNBS-modified peptide 103-118.

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**Purification on VYDAC 218TP54**

**Figure 9.** Purification of myoglobin from reaction mixture by reversed-phase HPLC. Column: VYDAC 218TP54, 5 µm, 300 Å, C18 (4.6 mm i.d. x 250 mm). Flow: 1.0 mL/min. Mobile phase: A = 5:95 ACN:water, 0.05% TFA. B = 55:45 ACN:water, 0.05% TFA. Gradient: Linear, 0% to 100% B over 20 minutes. Detection: UV absorbance with stream splitting (1:50) to MS (Fig. 10).
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*Also available in columns of 1.0 mm, 2.1 mm, and larger i.d.