Proteome Analysis Involving Off-Line 2-D LC of Intact Proteins, Proteolytic Digestion, and Capillary RP-LC-MS/MS Analysis Using Monolithic PS-DVB Columns

INTRODUCTION

The limitations of 2-D gel electrophoresis (GE) have stimulated the development of alternative techniques for large scale proteome analysis. More recently shotgun proteomics (i.e., multidimensional LC-MS/MS of digested complex protein samples) has been established as a robust technique for protein identification. This method is capable of identifying a large number of proteins, often on the basis of a few peptides alone. However, the detection and sequencing of low abundant peptides is impaired by the sample complexity and wide dynamic concentration.

The initial proteolytic digestion of the sample at the start of a shotgun procedure creates a highly complex peptide mixture with extreme concentration ratios from the high- and low-abundant proteins that were present in the sample. Peptides present in high levels throughout the fractionation prevent the detection of the peptides from proteins that were present at very low levels in the initial sample. Prefractionation of the intact proteins helps to separate high-abundant proteins from those present in small amounts. It also enriches the proteins into single, less complex fractions where much greater sequence coverage and, hence, confidence in the identifications can be achieved.

In this application note, an off-line multidimensional LC method combining ion-exchange and reversed-phase chromatography is described for separation of intact proteins followed by micropreparative fractionation and protein digestion. Protein digests are subsequently analyzed by capillary LC-MS/MS employing PS-DVB monolithic columns. The off-line 2-D LC method and LC-MS/MS analysis have been used for the separation and identification of proteins from human platelets.

EXPERIMENTAL

2-D LC of Proteins

Intact proteins were separated by following the workflow as shown in Figure 1. Proteins were separated on a ProPac® SAX column (2.0 mm x 25 cm). The mobile phase was collected in 1 min fractions in HPLC vials. Each ion-exchange fraction was subsequently injected onto a 500-µm i.d. PS-DVB monolithic column. The mobile phase from this column was collected in 24 one-minute fractions in a low-bind PCR 384 microtiter plate. After removal of the solvent, proteins were resuspended in ammonium carbonate buffer pH 8.0 with trypsin in a concentration of 5 ng/µL. The protein digest sample can be taken for immediate LC-MS/MS analysis or stored.

LC Conditions

LC system: UltiMate™ 3000, Probot™

First Dimension Separation (Ion-Exchange)

Human Platelet Proteins

Column:	ProPac SAX separation column, 2.0-mm i.d. × 25 cm, (P/N SP5554)
Solvent A:	10 mM Na_2HPO_4, pH 8
Solvent B:	10 mM Na_2HPO_4, pH 8, 1.25 M NaCl
Gradient:	0–630 mM NaCl in 20 min, 250 µL/min
Inj. Amount:	1 mg platelet proteins
Detection:	UV, 220 nm, 180-nL flow cell

Protein Second Dimension Separation (RP)

Column:	PS-DVB monolithic column 500-µm i.d. × 5 cm (60 ºC) (P/N 164087)
Solvent A:	Water, 0.05% TFA
Solvent B:	MeCN, 0.04% TFA
Gradient:	15–50% B in 20 min
Flow Rate:	20 µL/min
Samples:	IEX fractions of Platelets
Inj. Volume:	50–100 µL
Fractions:	Collected in 1-min fractions
Digestion:	Samples were evaporated in the 384 low-bind microtitre plate, 5 µL of sequencing grade trypsin (5 ng/µL) was added for an overnight digestion at room temperature.
Detection:	UV, 214 nm, 45-nL flow cell

Capillary LC-MS/MS Peptide Analysis

Column:	PS-DVB monolithic column 200-µm i.d. × 5 cm (60 ºC) (P/N 161409)
	Trap, PS-DVB monolithic, 200-µm i.d. × 5 mm (P/N 163972)
Eluent A:	Water, 0.05% TFA
Eluent B:	MeCN:water (50:50), 0.04% TFA
Samples:	Digested IEX protein fractions
Loading:	Water, 0.1% HFBA, 3 min, 20 µL/min
Gradient:	0–35% MeCN in 9 min, parallel LC (Figure 2)
Inj. Volume:	20 µL sample
Detection:	UV, 214 nm, 3-nL flow cell
	MS, Esquire 3000+ Ion Trap from Bruker Daltonics
	ESI-MS/MS, Positive ion mode, mass range 200–2000 *m/z*, cycle time 0.12 min
Protein ID:	MASCOT >2 peptides

RESULTS

Human Platelet Proteins

Intact human platelet proteins were separated and analyzed according to the workflow depicted in Figure 1. A typical separation of the human platelet proteins on the ProPac SAX column is shown in Figure 2A. Several high-abundant proteins are well separated, but it should be noted that the complexity of the sample is not reflected by the chromatogram; a large number of low-abundant proteins is present in the sample that are not resolved as single peaks. The peak eluting at 22 min is from the anionic detergent SDS, the peak at 2 min is from neutral detergent.

The ion-exchange fraction from 15 to 16 min was injected onto the 500-µm i.d. PS-DVB monolithic column to achieve a second-dimension separation. The chromatogram can be seen in Figure 2B. Although not all proteins are baseline resolved, fractions were collected and digested. The protein digests were analyzed by capillary LC-MS/MS analysis and the obtained MS/MS data was searched against the SWISS-PROT database. An example of a peptide separation, showing the BPC of the tryptic digest can be seen in Figure 2C.

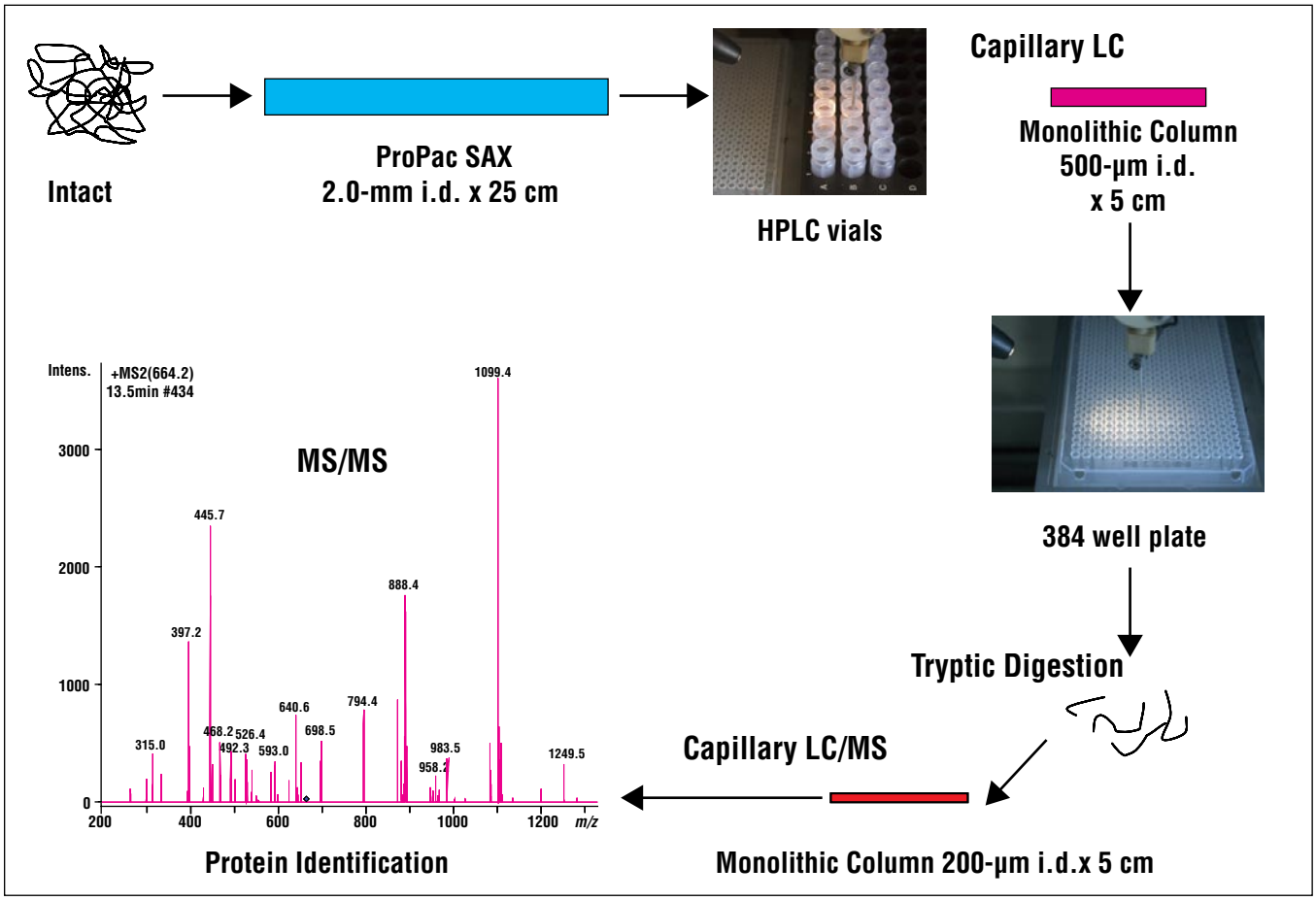

Figure 1. Multidimensional LC work flow.

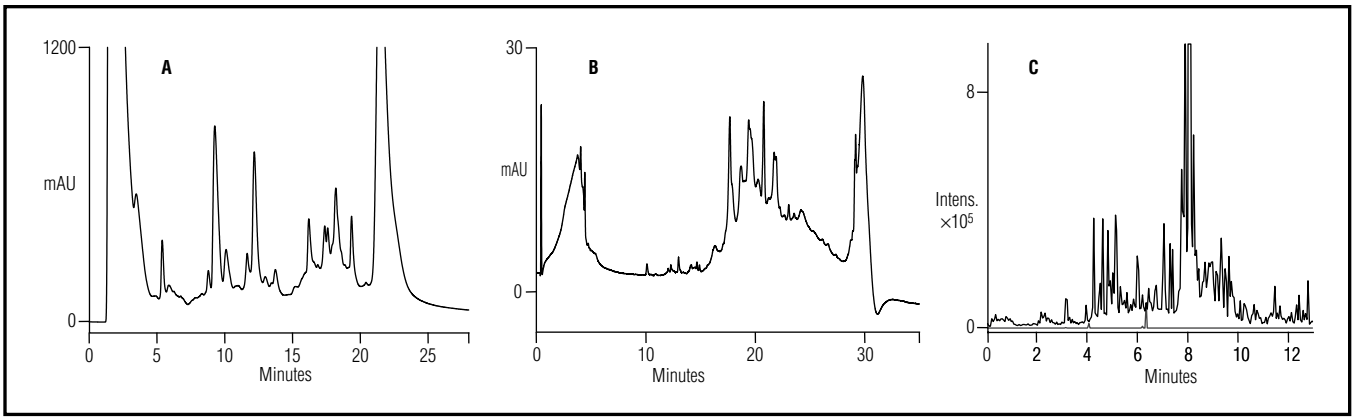

Figure 2. 2-D LC and MS/MS identification of human platelet proteins. (A) Separation of intact human platelet proteins on a ProPac SAX column (2.0-mm i.d. × 25 cm). (B) RP separation of SAX fraction 15–16 min on PS-DVB monolithic column (500-µm i.d. × 5 cm). (C) Base Peak Chromatogram MS and Extracted Ion Chromatogram of peptide with sequence LELLAAYEEVIR from Drebrin (Mass 1417.9 Da, observed ion 710.32+) on PS-DVB monolithic column (200-µm i.d. × 5 cm).

The peptide eluting at 6.3 min has the sequence LELLAAYEEVIR (Mass 1417.9 Da) and is identified as a peptide from Drebrin, a cytoplasmic actin-binding protein with a role in cell migration. A large number of platelet proteins were identified. A selection of proteins with *pI* and molecular mass extremes is shown in Table 1. The sequence coverage of identified proteins ranged from 2 to 40%.

The base peak chromatogram MS gives an indication of the high peak capacity of the monolithic capillary columns (200-µm i.d.) The peak capacity for the monolithic PS-DVB monolithic column is approximately 90 using a gradient of 9 min.

Table 1. Selection of Proteins Identified in Human Platelets by Top-down 2-D LC and Capillary LC-MS/MS

Protein	Accession No.	*pI*	M_W (Da)
Actin cross-linking family protein 7 (Macrophin)	Q9UPN3	5.27	620419
A kinase anchor protein 9	Q99996	4.95	453667
Ankyrin 2 (brain ankyrin)	Q01484	5.03	430344
Apolipoprotein B-100 [Precursor]	P04114	6.61	515563
Calmodulin	P02593	4.09	16706
Myosin regulatory light chain 2, smooth muscle isoform	P24844	4.80	19696
Tropomyosin beta chain	P06468	4.66	32851
Platelet basic protein precursor	P02775	9.04	13894
Platelet factor 4	P02776	8.93	10844
Protein kinase C inhibitor protein 1 (14-3-3 protein zeta/delta)	P29312	4.73	27745
Splicing factor, arginine/serine-rich 4	Q08170	11.52	56678
V(D)J recombination activating protein 1	P15918	8.96	119116

CONCLUSIONS

- The presented off-line 2-D LC method based on ProPac ion-exchange columns and PS-DVB monolithic columns offer high resolution separations of intact proteins.
- By this approach a number of cytoskeletal and signaling platelet proteins were identified in human platelets, some of which are important disease markers (V(D)J recombination activating protein 1, apolipoprotein B-100, myosin heavy chain type A, etc.).

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