PS-DVB Monolithic Columns Applied in an Off-Line 2-D LC/ESI-MS Bottom-Up Study for the Identification of Platelet Proteins

**INTRODUCTION**

For a long time, protein analysis relied on two-dimensional gel electrophoresis (2-D PAGE) as a dedicated technique for separating proteins from complex samples. To date, 2-D PAGE of proteins coupled to MALDI-MS is still the standard technique for proteome analysis. Despite the outstanding resolution and accuracy in protein identification, this technique shows several drawbacks. Most importantly are the limited dynamic range and the time-consuming protein analysis, which includes individual excision, digestion, and MS analysis of each resolved protein. A way to increase the throughput of this classical 2-D analysis method and remove a part of the sample-handling steps is to use a multidimensional LC/MS approach to separate peptides from digests of complex protein samples. In this study a packed strong cation-exchange column (SCX) was combined with a capillary PS-DVB Monolithic Trap and Separation Column in an off-line 2-D LC setup. Peptides from digested platelet proteins were separated on the SCX column, fractionated off-line, and subsequently analyzed by capillary LC MS/MS using Monolithic Columns.

**EXPERIMENTAL**

A tryptic digest of a human platelet sample was separated on a SCX column in the first dimension.

**First Dimension Separation (SCX)**

**LC System:** UltiMate™ 3000/WPS/GP 50 pump
**Probot™ Microfraction Collector**

**Column:** PolySULFOETHYL A™ column, 2.1 mm x 20 cm, 5 µm particles, 300 Å pore size, poly2-sulfoethyl aspartamide (PolyLC Inc., Columbia, MD)

**Buffers:**
- A: 5 mM NaH₂PO₄, pH 3.0
- B: as A + 1.0 M NaCl

**Flow Rate:** 250 µL/min

**Gradient:** 0–700 mM NaCl in 20 min

**Injected Amount:** 270 µg

**Fraction collection:** 1 min fractions

**Second Dimension Separation (RP)**

**LC system:** UltiMate 3000
**Sample:** 28 SCX fractions

772 ng platelet protein digest loaded on the column, on average

**MS:** Esquire 3000 + Ion Trap from Bruker Daltonics
**Monolithic Capillary Column**

Column: PS-DVB Monolithic Column, 200 μm i.d. x 5 cm (P/N 161409)

Trap column: PS-DVB Monolithic, 200 μm i.d. x 0.5 cm (P/N 163972)

Temperature: 60 ºC

Flow-Rate: 2.7 μL/min

Gradient: 0–40% ACN in 10 min and 0–35% ACN in 25 min

Flow Cell: 3 nL

Sample Loading: H₂O, 0.05% TFA, 3 min at 20 μL/min

Elution Solvents: A: H₂O, 0.05% TFA

B: ACN/H₂O, 50:50, 0.04% TFA

Inj. Volume: 20 μL

**ESI-MS/MS Settings**

Ion Mode: Positive

Mass Range: 200–2000 m/z

Cycle Time: 0.12 min

**ESI-MS/MS Spectra Analysis**

Search Parameters

Mass tolerance of 1.0 Da for the parent ion, 0.5 Da for the fragment ions Protein IDs defined by at least two peptides and peptide ion score ≥14.

**RESULTS**

**SCX Separation of Human Blood Platelet Peptides—First Dimension**

The off-line 2-D LC method was optimized in both dimensions for optimal sample analysis as well as reduced analysis time compared to 2-D gels. Peptide separation on the poly2-sulfoethyl aspartamide SCX column was optimized to reduce breakthrough of peptides by applying a linear gradient that started with a low salt concentration, followed by a relatively long equilibration time, as shown in Figure 2. Twenty-eight fractions were automatically collected with the microfraction collector. Each SCX fraction was subsequently preconcentrated on a Monolithic Trap Column and then separated on a Monolithic Capillary Column.

**Reversed Phase Separation of Human Blood Platelet Peptides—Second Dimension**

Figure 3 shows the chromatograms of the reversed phase separation on the Monolithic Column switching setup for 12 consecutive fractions (9–20). In multidimensional LC methods it is of the utmost importance that the recurrence of peptides in adjacent fractions is minimal to prevent “peak dilution”. The distribution of peptides was evaluated for the SCX fractions upon separation on a Monolithic Column in the second dimension, with a long gradient (0–35% MeCN in 25 min) by examination of the MS signals.
The absence of peptide recurrence or crossover in consecutive SCX fractions enables accurate and reliable peptide separation in the second dimension (RP). No peptides were found in more than two SCX fractions. This ensures a peak intensity high enough to be detected by the mass spectrometer, which is especially important for low abundant peptides originating from low abundance proteins. Two peptides coming from integrin \( \alpha \)-2b (masses 1270.69 Da and 1501.83 Da) are found only in fraction 15. In spite of their low intensities (6717 and 6659 counts, respectively), the peaks were unambiguously identified as integrin peptides. This demonstrates the high efficiency of the SCX column during gradient separations.

**Figure 3. Second dimension, 2-D LC—Fractions 9–20 from the optimized SCX run, separated on a Monolithic Capillary Column with column switching on a Monolithic Trap Column.**

**Figure 4. Reproducibility of SCX fraction 15 coming from two SCX runs, separated by RP-LC on a Monolithic Capillary Column.**

**Reproducibility of the Off-Line 2-D LC Method**

The reproducibility of the off-line 2-D LC method was evaluated by replicate experiments. The method involves separation on a 2.0 mm i.d. packed column, fractionation, and re-injection on the Monolithic Capillary Column switching setup. It was found that the method was reproducible in terms of the chromatographic parameters. As shown in Figure 4, the replicate fraction 15 from the two SCX runs are reproducible in their UV trace, the difference between the retention times of each peak between runs is <1 s. The proteins identified in the two number 15 fractions coming from different SCX runs show 80% overlap. When the combined data from all fractions of two SCX runs are compared, the overlap of the total number of proteins found in the two sets of SCX fractions is around 70%.
**Identification of Proteins**

By applying the off-line 2-D LC technique involving Monolithic Columns, 151 proteins were identified in a human platelet sample.

In terms of sequence coverage, high abundance platelet proteins (platelet factor 4, platelet basic protein precursor, plekstrin) and cytoskeleton proteins (actin, myosin) were found with high sequence coverage values, varying from 15 to 40%. Most proteins were found and represented by low values (2–10%), including low abundance proteins. However, replicate measurements have confirmed the identity of protein hits with low sequence coverage (protocadherin α-C1 precursor, zyxin, macrophin). Some of the proteins identified show molecular mass extremes. The protein with the highest mass of 620418 Da was macrophin (actin cross-linking family protein 7) while platelet factor 4 had the lowest molecular mass, 10844 Da. The proposed 2-D LC methods lead to the identification of proteins with a wide range in pI and molecular weight. The protein with the lowest pI was Tropomyosin β-chain, pI 4.66, while splicing factor, arginine/serine-rich 4 had the highest pI value of 11.52. Some of the proteins with pI and molecular mass extremes are indicated in Table 1.

From the membrane protein group, the method lead to the identification of various proteins including adenyl cyclase-associated protein 1, several integrins, and voltage-dependent channel proteins. These three proteins were the most commonly observed throughout the replicate analyses.

**Table 1. Selection from the 151 Proteins Identified by RP-LC Separation on a Monolithic Capillary Column—pI and Molecular Weight Information**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>pI</th>
<th>Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin cross-linking family protein 7 (Macrophin)</td>
<td>Q9UPN3</td>
<td>5.27</td>
<td>620419</td>
</tr>
<tr>
<td>Ankyrin 2 (brain ankyrin)</td>
<td>Q01484</td>
<td>5.03</td>
<td>430344</td>
</tr>
<tr>
<td>Apolipoprotein B-100 [Precursor]</td>
<td>P04114</td>
<td>6.61</td>
<td>515563</td>
</tr>
<tr>
<td>Collagen α-6 (IV) chain precursor</td>
<td>Q14031</td>
<td>9.41</td>
<td>163797</td>
</tr>
<tr>
<td>Endoplasmin [Precursor]</td>
<td>P14625</td>
<td>4.76</td>
<td>92469</td>
</tr>
<tr>
<td>Huntingtin (Huntington's disease protein)</td>
<td>P42858</td>
<td>5.81</td>
<td>347860</td>
</tr>
<tr>
<td>Myosin regulatory light chain 2, smooth muscle isoform</td>
<td>P24844</td>
<td>4.80</td>
<td>19696</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>P02776</td>
<td>8.93</td>
<td>10844</td>
</tr>
<tr>
<td>Protein kinase C inhibitor protein-1 (14-3-3 protein zeta/delta)</td>
<td>P29312</td>
<td>4.73</td>
<td>27745</td>
</tr>
<tr>
<td>Splicing factor, arginine/serine-rich 4</td>
<td>Q08170</td>
<td>11.52</td>
<td>56678</td>
</tr>
<tr>
<td>Tropomyosin β–chain</td>
<td>P06468</td>
<td>4.66</td>
<td>32851</td>
</tr>
<tr>
<td>Utrophin (Dystrophin-related protein 1)</td>
<td>P46939</td>
<td>5.21</td>
<td>394494</td>
</tr>
<tr>
<td>Vasopressin V1b receptor</td>
<td>P47901</td>
<td>9.21</td>
<td>46971</td>
</tr>
</tbody>
</table>
The identification of a low abundant peptide from transgelin is illustrated in Figure 5. The peptide is eluting at 23.5 min as indicated.

A. UV chromatogram of RP separation of SCX fraction 13 on a Monolithic Capillary Column. Peptide from transgelin DGTVCELINALYPEGQAPVK is eluting at $t = 23.5$ min.

B. Base Peak Chromatogram MS and Extracted Ion Chromatogram 763.5$^+$.

C. Base Peak Chromatogram MS/MS and Extracted Ion Chromatogram 763.5$^+$.

D. MS/MS spectrum of DGTVCELINALYPEGQAPVK.

Figure 5. Identification of transgelin peptide with sequence DGTVCELINALYPEGQAPVK (mass 2287.14 Da, observed ion 763.5$^+$).
Influence of Gradient Conditions on Peptide Identification

A test was performed for the same fraction (15) separated in the second dimension with two gradients: a steep gradient (0–40% MeCN in 10 min) and a shallow gradient (0–35% MeCN in 25 min). It is important to see whether the gradient slope influences the number of proteins identified in the fraction. By summing up the significant hits from five replicate runs of fraction 15 with the steep gradient, a total of 15 proteins were identified, with an average number of six proteins in each run. The flat gradient lead to the identification of a total of 16 proteins, obtained from five replicate runs, with an average number of 11 proteins in each run. This indicates that the application of shallow gradients leads to the identification of more proteins.

The proteins found with the two gradients are partly different, which indicates that repeating the peptide separation for each fraction with different gradients can improve the number of identified proteins. Performing consecutive runs with the same gradient for each fraction (data not shown) supported this idea.

CONCLUSIONS

1. The off-line 2-D LC setup presented in combination with automatic fraction collection is reproducible, efficient, and easy to handle, at the same time offering high analysis throughput.
2. Monolithic Capillary Trap and Separation Columns in a column switching setup can be used for the analysis of complex proteomic samples.
3. One peptide is generally found in not more than two consecutive fractions, showing the efficiency of the SCX separations.
4. The off-line 2-D LC method allowed the identification of 151 platelet proteins, such as Platelet basic protein, Pleckstrin, β-Thromboglobulin, Thrombospondin, as well as low abundance proteins (Zyxin).

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