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# Top-down proteomic analysis by use of molecular weight-based separation and online LC-FTMS

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***Introduction***

Recently, we developed and implemented an intact protein separation scheme based on molecular weight known as gel-eluted liquid fraction entrapment electrophoresis (GELFrEE) as the first dimensional protein fractionation.[1,2] This molecular weight-based separation involves continuous elution SDS-PAGE in a tube format, in which proteins are constantly eluted from the gel column and collected in the solution phase, fractionating proteins from 5 kDa to 200 kDa in 1.5 h.[3] GELFrEE provided broad mass range separation with high-resolution, reproducibility, and recovery, facilitating high-throughput proteomic analysis. GELFrEE fractions generated from HeLa S3 cells were successfully analyzed by online nanocapillary reversed-phase liquid chromatography (RPLC) with 12 Tesla Fourier transform mass spectrometry (FTMS) based on top-down approach. Based on our previous results for proteomic analysis, the use of GELFrEE followed by online RPLC and Orbitrap mass spectrometer with ultra-high resolution is expected to establish a high-throughput method of analyzing the peptide hormones and protein drugs covering wide molecular weight ranges. Currently, we are analyzing protein standard mixtures from 5 to 30 kDa based on top-down approach by use of online RPLC followed by Orbitrap mass spectrometer and optimizing the instrumental parameters in order to differentiate the protein drugs from endogenous forms. We will apply GELFrEE scheme to fractionate the substances present in urine samples and analyze the spiked protein drugs by use of Orbitrap mass spectrometer as the next step, thus ultimately aiming to establish a robust method of peptide and protein drugs based on MS.

***Methods***

1. GELFrEE: Tube gels with tris-glycine or tris-tricine gel media were cast to 15% T for the resolving and 4% T for the stacking gels. Approximately 100 µL of sample was loaded onto a tube gel column and separation occurred with a constant application of 240 V. 16-18 GELFrEE fractions with a volume of 150 µL each were collected over 1.5 h after elution of the dye front.

2. LC-MS/MS analysis: GELFrEE fractions were cleaned to remove SDS by chloroform-methanol precipitation method and separated on a C4 or PLRP-S analytical column (75 µm x 10 cm, 5 µm particle size, New Objective, Inc., Woburn, MA, USA) at 300 nL/min from an Eksigent 1D Plus system. The samples were then analyzed by use of 12 T LTQ-FT Ultra mass spectrometer for which the parameters were a data-dependent top 2 MS/MS acquisition with *m/z* 10 isolation window, 8 microscans at 180 K resolving power ($m/\Delta m_{50\%}$, in which $\Delta m_{50\%}$ is mass spectral peak full width at half-maximum peak height) in the MS scan event (*m/z* 500-2000 scan range) and 8 microscans at 60 K resolving power in the MS/MS scan events (scan ranges from one third of the precursor *m/z* to *m/z* 2000). Dynamic exclusion was enabled with a repeat count of 1, an exclusion duration of 5000 s, and a repeat duration of 240 s.

3. Standard protein mixture analysis: 5 standard protein mixtures consisting of insulins originated both from human and porcine, cytochrome C, myoglobin, and carbonic anhydrase were prepared as 20 nmol/mL in 0.2% formic acid. For mass spectral analysis for intact protein measurement, a total of 5 µL of standard protein mixture solution was injected and separated on a C4 analytical column (2.1 mm x 10 cm, 3 µm particle size, Thermo Fisher Scientific, San Jose, CA) at 200 µL/min from a Surveyor LC pump. For performing collisional-induced fragmentation (CID) for insulins from human and bovine, 1 µL of 20 nmol/mL of insulin mixtures was injected and separated on a C4 analytical column. The standard protein samples were then analyzed by use of Orbitrap mass spectrometer. Full MS scans ranging from *m/z* 300 to 2000 were recorded at 60 K resolving power, and MS/MS scans were acquired at 30 K resolving power.

***Results***

The GELFrEE fractions containing up to 25 kDa generated from both human HeLa S3 cells and mouse B16F10 cells were analyzed by online nanocapillary reversed-phase liquid chromatography (RPLC) with 12 T FTMS. Single injections gave approximately 50-60 detectable proteins, about half of which were identified with PTM information.

Figure 1 represents an example of protein separation by use of online nanocapillary RPLC coupled to FTMS from a GELFrEE fraction including 13 to 18 kDa of proteins.

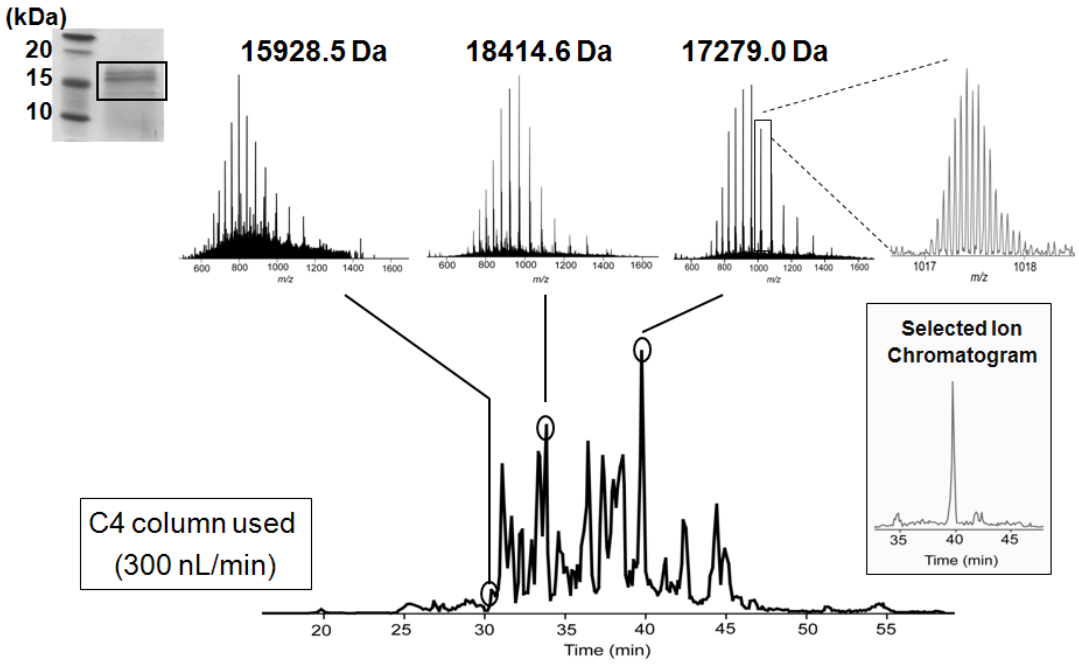


**Figure 1**. Online intact protein separation and detection generated from a GELFrEE fraction containing proteins with molecular weight from 13 to 18 kDa

Since baseline-resolved mass spectral data was acquired from high-resolution mass spectrometry, the molecular weight of intact proteins was able to be determined accurately and the protein isoforms with similar molecular weight ranges were able to be detected and characterized.

While GELFrEE and Orbitrap mass spectrometer with high-resolution are expected to provide a high-throughput format of protein drug analysis, we first tested whether we can detect and differentiate standard protein mixtures with molecular ranges from 5 to 30 kDa by use of Orbitrap mass spectrometer. While the full MS spectrum seen in Figure 2 was obtained from the injection of 100 pmole of standard protein mixtures, the standard proteins except carbonic anhydrase were detected down to 2 pmole of injection with accurate molecular weight information.

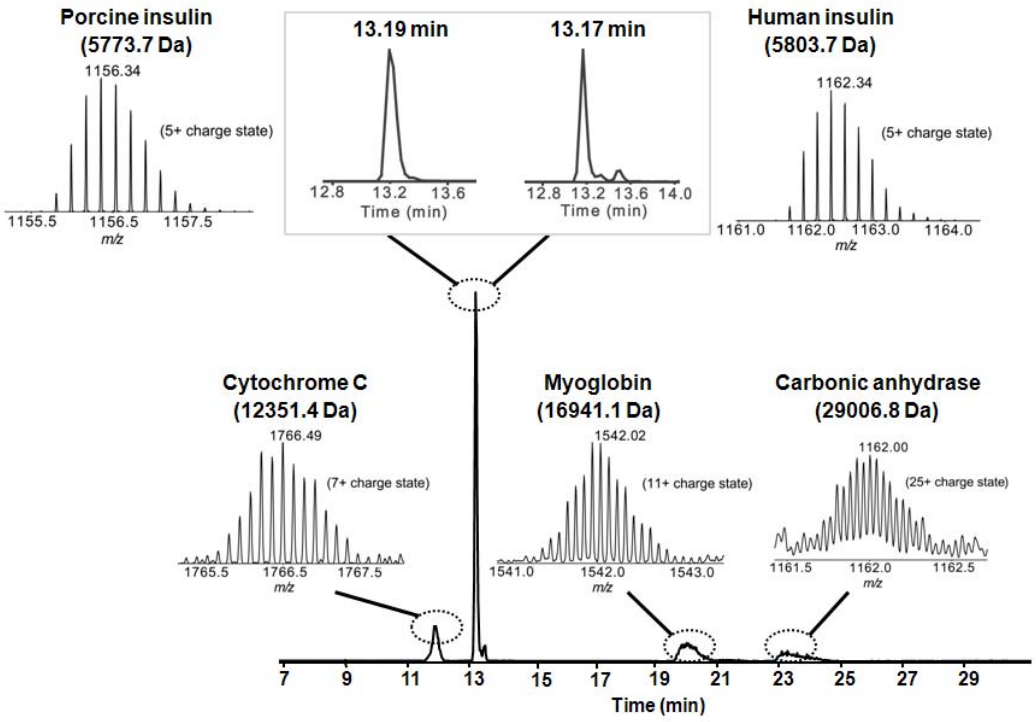


**Figure 2.** Online protein analysis by use of LC-MS analysis

We further tested if we could differentiate the protein analogs with very similar amino acid sequences based on tandem MS fragmentation. Figure 3 illustrates both MS and MS/MS spectra generated from porcine insulin and human insulin, respectively, in which two protein analogs have only one different amino acid. In Figure 3, MS/MS spectra were generated from CID fragmentation of the sixfold protonated precursor ions for each insulin, which are *m/z* 963.28 and 968.28 of monoisotopic peaks from porcine and human, respectively. Due to the characteristic fragment ions from the two insulin analogs, they were able to be characterized and differentiated as observed from the recent studies by Thevis *et al.* for insulin analysis.[4-6] As the next step, we will seek to apply GELFrEE scheme to fractionate the substances present in urine samples and analyze the spiked protein drugs including insulins by use of Orbitrap mass spectrometer with high-resolution.

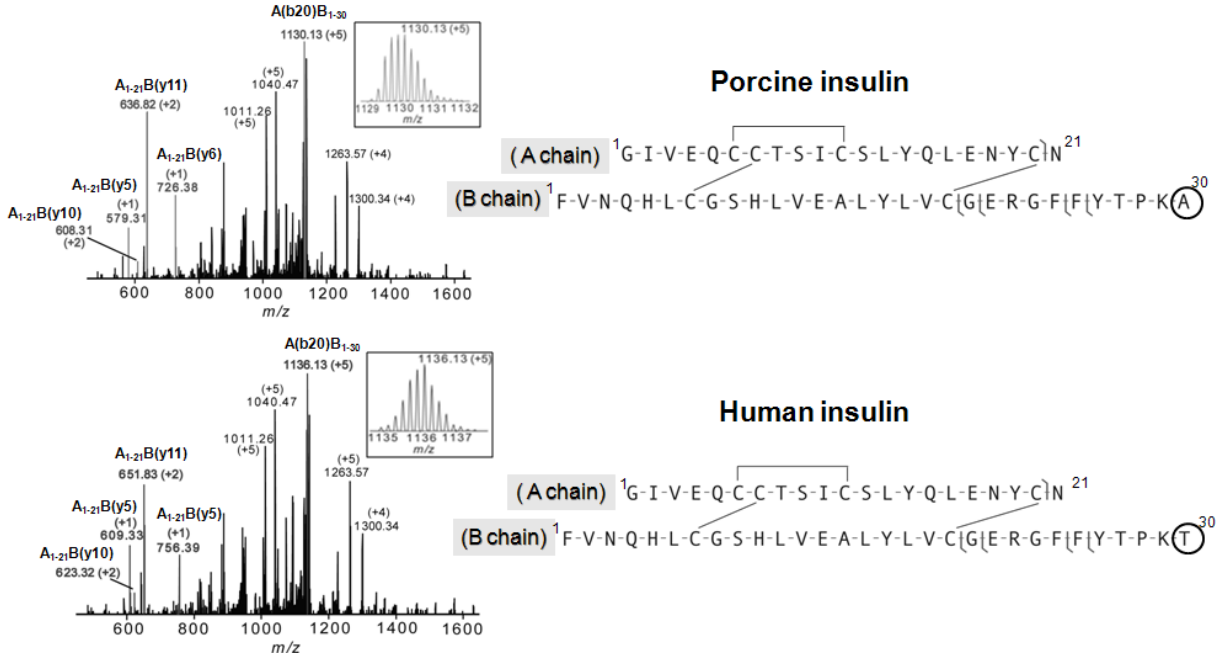


**Figure 3.** Characterization and differentiation of two insulin analogs by use of high-resolution Orbitrap mass spectrometer

## *Conclusion*

In our study, we were able to demonstrate that GELFrEE could be employed as a robust method of intact protein separation for high-throughput MS analysis, and intact protein analysis by use of high-resolution FTMS and Oribtrap mass spectrometer facilitated detection of different protein isoforms. Therefore, we believe that GELFrEE and high-resolution Orbitrap mass spectrometer can be applied to analyze the peptide hormones and protein drugs covering wide molecular weight ranges that were prohibited in amateur or elite sports for their high-throughput analysis.

## *References*

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