Mass Spectrometry Based Proteomics

Toward understanding neuropathic pain

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Introduction

1. Mass spectrometry based proteomics

A term “proteomics” was first introduced in 1997 by Peter James, as the study of the proteome (the total protein complement of a genome) (Wilkins et al, 1996). Proteomics is a broad field, and the different branches (Figure 1) devoted to different purposes and/or characterized by specific detection methods are used in every branch. Proteomics can also be characterized as a high throughput holistic science which answers which proteins, where, and when are expressed.

Nowadays, the number of proteins that can be identified in a single sample can reach a few thousands. The reason behind this is the increasing usage of mass spectrometers which are gradually replacing the gel-based method. Mass spectrometry is an analytical technique that is used to identify a compound by measuring the mass-to-charge ratio of produced ions (as described below). As proteins normally form complex mixtures, often the mass spectrometer is connected online or off-line (rarer) to a reverse phase HPLC (High-performance liquid chromatography) as an upfront separation system.

**How mass spectrometry works**

A mass spectrometer consists of three parts: an ion source, a mass analyzer (or several analyzers) and a detector, as shown in Figure 2. There are many different types of mass spectrometers depending on the combination of all different parts. Still, the main operational principle for all of them remains the same; ionization of a protein/peptide mixture to generate charged molecular ions with or without fragments and measuring their mass-to-charge ratios.
There are two different approaches that can be used for proteins characterization, top-down and bottom-up proteomics\(^1\). The difference between these two methods is that in top-down the entire protein is introduced in the mass spectrometer, while in the bottom-up, digests of the proteins are introduced to the mass spectrometer.

As reference for further introduction to the instrumentation of a mass spectrometer, *Orbitrap Velos Pro* by Thermo Scientific will be used.

**Ion source.** The ion source is responsible for the transformation of the polypeptides to gas-phase ions, which will be transmitted to the mass analyzer. Proteins are quite sensitive molecules, for this reason soft ionization methods are preferred. One of them is Electrospray Ionization (ESI), where the ions are produced by an electric field, as shown in Figure 3. As the sample exits the capillary and due to the high voltage between the capillary and the inlet of the mass spectrometer, an aerosol of charged droplets is formed. (Yamashita and Fenn 1997). Then, the ions initially enter the inlet and follow their way to the mass analyzer.

**Mass analyzer.** The mass analyzer is the most important part of a mass spectrometer as it is the one responsible for separating the previously ionized peptides according to their mass to charge ratio (m/z). There are several types of mass analyzers that differ in mass accuracy and resolution. Orbitraps were first introduced in 1994 and they offer higher resolution and mass accuracy compared to prior mass analyzers\(^1\). In an orbitrap, the ions are trapped in the electric field moving around and along a central electrode (oscillation movement), as shown in Figure 4. (Makarov 2000, Kaufmann 2010)

\(^1\)Here we are focusing in bottom-up proteomics.
Detector. The detector is the last part of the set up. The oscillation movement of the ions, that previously were moving along and around the orbitrap, generates an image current in the detector plates. This image current is recorded by the detector, which then transforms it into a mass spectrum, using Fourier transformation. (Dubois 1999)

Protein quantification - Stable isotope dimethyl labeling

In proteomics one of the most frequent questions is what is the difference in the proteome between two or more different states. Mass spectrometry can give the answer to this question. It is not only a powerful tool in the aspect of quantification, but also offers very high sensitivity and selectivity compared to other quantification techniques (Boersema JP et al. 2009). Stable isotope dimethyl labeling (Figure 6) is a quite recent label technique and is based on introducing a dimethyl group in the peptide’s amine (-NH₂) groups after trypsination. There are three different dimethyl groups that can been added; –CH₃, -CHD₂ and ¹³CHD₂, which give a light, a medium and a heavy label respectively. This gives a mass difference of 4 Da between differentially dimethyl labeled peptides. Intensities of the peaks corresponding to differentially labeled peptides are used for relative quantification.

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Figure 5 schematic representation of stable isotopic dimethyl labeling procedure
2. Neuropathic pain

Neuropathic pain is a specific category of chronic pain (Champbell and Meyer 2006), caused by the dysfunction of sensory nervous system. More specific, it can be caused either by injury (either in central or peripheral nervous system), or numerous other conditions, such as stroke, HIV, cancer, diabetes, multiple sclerosis, and so on (Woolf and Mannion 1999). Diagnose is difficult and is based mostly in patient’s description of the pain. They usually describe it as shooting and burning pain, or itchy, hot and many more. There are some tests that can identify neuronal trauma, but there are also cases that the test can not explain the pain. There is no universal treatment for neuropathic pain, but different categories of medication can be used, depending on the cause of the pain. For this reason, understanding the basic pathophysiology of neuropathic pain is needed.

Many animal models have been developed to understand the pathophysiology of neuropathic pain, with rats been one of the most frequently used. One of the most common way to mimic neuropathic pain in rats is injure some of the peripheral nerves as shown in Figure 7. Injury in different parts of the nerve can cause different clinical symptoms. The spinal nerve ligation model (SNL) was first reported in 1992 by Kim and Chung. In this model both L5 and L6 nerves are ligated. The ligation can either be done in the left or the right side of the body. A ligation in spinal nerve causes a miscommunication between the peripheral nervous system and the brain, which subsequently can cause different forms of neuropathic pain.

In these type of studies is often to use two different sets of control samples (Kim 1997). The first one is the Naive rats, in which no operation have been done and are healthy wild type rats. The other category is Sham rats, which are rats that they have been operated in the same way as SNL ones, but ligation has not been occurred. In this way, differences from actual cause of pain can be monitored and differences from the operation itself can been excluded.

AIM

The aim of this project was to get insight into mass spectrometry based proteomics, get familiariized with novel techniques, and obtain the operating skills with modern Orbitrap mass spectrometers. In order to achieve this, the proteome changes in neuropathic pain responses corresponding to nerve injury side in individual rat’s spinal cord were explored. We focused in protein identification and quantification of the expressed proteins in 3 different set of samples, SNL, Sham and Naive rats.
Material and methods

Sample preparation

For this study, three different groups of spinal nerve ligation (SNL), Sham-operated (operated rats without nerve ligation) and Naive rats were used. There were 10 individuals in each group. All procedures in animal handling and preparation, as well as protein extraction were previously performed as described by Sui et al., 2014. The protein extracts were stored in the buffer containing 8 M urea and 1% octyl-β-D-glucopyranoside in 10 mM Tris, pH 8.

Bradford assay

The protein concentration for all the samples was measured by using the Bradford assay with Bio-Rad reagents. The standard protocol suggested by the manufactory was used. Bovine serum albumin was used to for preparing the standard curve and the absorbance was measured at 595 nm.

Protein digestion

0.1 M TEAB (triethylammonium bicarbonate) was added to 20µg of extracted proteins to a final volume of 50 µL. The samples were reduced with 10 µL of 15mM dithiothreitol at 56°C for 30 min, and alkylated with 10µL of 33 mM iodoacetamide in the dark for 30 min. Then, we either followed:

1. In solution digestion. 1 µg of trypsin/Lys-C mixture was added to the samples and incubation at 37°C overnight followed. The next day, acetic acid was added and the samples were dried. 50 µL of 0.1% formic acid was added into dried samples, and reconstituted samples were mixed with 1mL water saturated ethyl acetate in order to remove beta-octyl glucopyranoside. Upper organic layer was then removed and the same procedure was repeated two more times. Finally, the samples were desalted using C-18 SPE columns and dried once again.

2. On filter digestion. The samples were transferred into 3 kDa cutoff filters and centrifuged at 14000 g for 15 min (all centrifugations were further performed at the same conditions). Then, three buffer exchanges were followed with: i) 50 µL of 2% acetonitrile in 100 mM TEAB, ii) 50 µL of 50% acetonitrile in 100 mM TEAB and iii) 50 µL of 100 mM TEAB. Trypsination was followed by adding 1 µg of trypsin/Lys-C mixture in 50 µL of 100 mM TEAB at 37°C for 90 min. The samples were then centrifuged and the eluates were collected. 50 µL of 0.1% formic acid were added to the filters which were spun down once again. The combined eluates were dried.
**Dimethyl labeling**

The procedure followed for dimethyl labeling was the same as described by Boersema et al. Tryptic peptides from the dorsal side of Naive rat’s spinal cord were labeled as heavy. Left sham ipsi dorsal and left SNL contra dorsal tryptic peptides were labeled as medium. Left sham contra dorsal and left SNL ipsi dorsal tryptic peptides were labeled as light. After labeling the following labeled samples were mixed together: i) Naive dorsal, left sham ipsi dorsal and left sham contra dorsal and ii) Naive dorsal, left SNL ipsi dorsal and left SNL contra dorsal. After mixing the samples were desalted using C-18 SPE columns, dried and stored at -80°C.

**Nano LC-MS/MS and evaluation**

1µg of each combined differentially labeled sample was dissolved in 5 µL of 0.1% Formic Acid (FA) and then was loaded in Thermo Scientific EASY-nLC 1000, which was connected to Thermo Scientific Orbitrap Velos Pro mass spectrometer.

The separation was carried out on a reverse phase C18 nanocolumn. Elution is occurred by gradient increase of acetonitrile from 4% to 50% in 40 min at flow rate of 0.2 µL/min. Mass spectrometric analysis was performed in CID fragmentation mode and data dependent acquisition as described elsewhere Sundberg et.al Biochemistry and Biophysics reports 3 (2015) 68-75. MaxQuant software (Cox and Mann 2008) was used for identification, quantification and evaluation of the data extracted from then Thermo Scientific Orbitrap Velos Pro.

**Results and discussion**

In our project we have 5 different sample groups, as shown in Figure 7. The first group of samples is the control group of naive rats. For our experiment we used segments from the dorsal side of spinal cord, both from the left and the right side (Figure 8a). This is the only group where the protein extracts from individual rats’ spinal cords were pooled together. The number of individual rats used was 30. For the rest of the groups, the segments of spinal cord adjacent to which the ligation (for SNL) or the surgery (for Sham) was performed are shown in pink color, while the segments we used for the experiments are shown in green circles. For these groups each sample represents individual rat.
For our first experiment we used SNL rats that had the ligation in left part of the spinal cord (Figure 8b, c), as well as naive-control rats. In this experiment we wanted to explore if there is a difference between the ipsi (the actual side that the ligation happened) and the contra (the opposite side from the one that the ligation happened) side of the ligation in the dorsal side of the spinal cord. 10 biological replicates were prepared, each of them contained 5 µg of the naive pool, 5 µg the left SNL ipsi dorsal individual and 5 µg the left SNL contra dorsal individual.

For our second experiment, we used Sham rats, which had been operated in the left side of the spinal cord. Corresponding to the first experiment, we prepared 10 biological replicates, each of them containing 5 µg of the naive pool, 5 µg the left Sham ipsi dorsal individual and 5 µg the left Sham contra dorsal individual.

In our first attempt to run the labeled samples, only three of them gave us results. The reason behind this is that the LC-MS system stopped due to overpressure. These three samples gave us 500 quantified proteins out of 700 that had been identified in total. In Table 1 a set of the neuropathic pain associated proteins is shown. The same set of proteins have been previously described by Sui et al. in 2014. Several other attempts followed, but in none of them we had close results. In the majority of them we could maximum quantify 300 proteins of a total of 500 proteins. It is important to be stated that single results can not be considered as significant and this is the reason that we do not have any definite conclusion.

Several things were tried in order to obtain results as good as the ones mentioned before:

- We added a heater in the column in order to avoid overpressure.
- We changed several columns, both packed in house and commercial ones. The possible reason behind this is aging of packing material could have occurred after several dozens of samples.
- We used another LC/MS system for these three samples in order to confirm the results, but we never did.
- We went back to the initial individual samples and that time we digested them in solution. The reason behind this, is that we observed that some of the filters used for digesting the samples the first time were cracked. Even then, we did not have any better result (even for the ones that gave us 500 quantified proteins).

Figure 8 schematic overview of the spinal cord segmentation. D stands for dorsal side and V for ventral. I stand for ipsi side, while C for contra side
In conclusion, we do not have a clear picture of what the actual problem was. One of the main suspicions is that something went wrong with either the LC system or the mass spectrometer itself. In the period that the experiments were held, we faced several problems with both of the machines and their efficiency. Also, one other explanation could be that the samples were kept for relatively long time or something in the whole procedure made them to degrade during the period we were trying to find what was wrong with them. Further investigation is needed in order to confirm what went wrong in the procedure.

Despite the fact that no actual conclusions drawn through the exploration of the proteome change in neuropathic pain, the important aims of familiarization with the mass spectrometry based proteomics and the independent operation of the handling techniques were achieved.

*Table 1* The difference of the protein expression of neuropathic associated proteins between Naive rats (H-heavy labeled) and either ipsi (M-medium labeled) or contra (L-light labeled) lesions of left side operated Sham rats.

<table>
<thead>
<tr>
<th>UniProt ID</th>
<th>M/L ratio</th>
<th>H/L ratio</th>
<th>H/M ratio</th>
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<tbody>
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<td>1433B_RAT</td>
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The project took place in the department of Chemistry, Biomedical Center (BMC), Uppsala University, and was completed after a four-month period (July-October 2016). The main goal was the familiarization with the field of mass spectrometry based proteomics. For this reason, an introduction of the instrumentation and sample preparation was held for a period of two weeks. After that, independent lab work was mostly carried. Apart from Konstantin Artemenko, who was the main supervisor, all the members of the neurochemistry group were helpful and supportive with all the problems that came up during the project period. Unfortunately, due to an unexpected problem with the instruments, the actual samples have not been analyzed. Although, this fact was a great opportunity for deeply understand the whole procedure by trying to find the proper solution.

Reference