Lab-on-a-tissue : optimization of on-tissue chemistry for improved mass spectrometry imaging

Heijs, B.P.A.M.

Chapter 2

Brain region-specific dynamics of on-tissue protein digestion using MALDI-MSI

Bram Heijs; Else A. Tolner; Judith V.M.G. Bovée; Arn M.J.M. van den Maagdenberg; Liam A. McDonnell, "Brain region-specific dynamics of on-tissue protein digestion using MALDI-MSI," *Journal of Proteome Research* 14(12), pp. 5348-5354, 2015.

In MSI, on-tissue proteolytic digestion is performed to access larger protein species and to assign protein identities through matching the detected peaks with those obtained by LC-MS/MS analyses of tissue extracts. The on-tissue proteolytic digestion also allows the analysis of proteins from FFPE tissues. For these reasons on-tissue digestion-based MSI is frequently used in clinical investigations, e.g. to determine changes in protein content and distribution associated with disease. In this work we sought to investigate the completeness and uniformity of the digestion in on-tissue digestion MSI. Based on an extensive experiment investigating three groups with varying incubation times: (i) 1.5 hours, (ii) 3 hours, and (iii) 18 hours, we have found that longer incubation times improve the repeatability of the analyses. Furthermore, we discovered morphology-associated differences in the completeness of the proteolysis for short incubation times. These results support the notion that a more complete proteolysis allows better quantitation.

2.1 Introduction

Since its introduction in 1997 by Caprioli *et al.* (Caprioli et al., 1997) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) has become a valuable tool in clinical research. MALDI-MSI has the ability of simultaneously recording hundreds of untargeted biomolecular analytes in a spatially correlated manner to provide the analyst with cell type-specific molecular signatures. Different sample preparation strategies can be applied to investigate the abundance and spatial distribution of various molecular classes, ranging from endogenous small molecules and pharmaceuticals, to lipids, peptides, and proteins, directly from a tissue section (McDonnell and Heeren, 2007). The aforementioned characteristics make MALDI-MSI a well-suited tool for biomarker discovery or for the investigation of the molecular mechanisms underlying disease (Balluff et al., 2011; Carreira et al., 2015; Cole et al., 2011; Djidja et al., 2010; Poté et al., 2013).

In order to identify proteins, increase proteome coverage and enable the analysis of proteins from formalin-fixed paraffin embedded tissue sections, the enzymatic digestion performed in classic bottom-up proteomics has been adapted to MALDI-MSI and is also mostly performed using the endopeptidase trypsin (Groseclose et al., 2008; Heijs et al., 2015; Lemaire et al., 2006; Shimma et al., 2006).

As discussed by Brownridge *et al.* (Brownridge and Beynon, 2011), for *in solution* bottom-up proteomics, the digestion of a protein to its 'limit peptides' can follow many pathways, resulting in a large number of intermediate digestion products. In this context, 'limit peptides' are proteolytic peptides that lack missed proteolytic cleavage sites, meaning that all peptide bonds that *could* be cleaved *were* cleaved. It can be argued that the repeatability of an enzymatic digestion is solely achieved and only guaranteed if all proteins are converted to their limit peptides. In the same study it was shown that not all amino acid sequences have the same digestion efficiency, and it was

concluded that enzyme incubation time is key to achieving repeatable digestion results (Brownridge and Beynon, 2011).

A tissue section can be very heterogeneous and constitutes a chemically complex environment. Accordingly, there are questions pertaining to the efficiency of the proteolysis and whether there may be region-specific differences in reaction rate, both of which would affect the repeatability of on-tissue proteolytic digestion MSI experiments. Surprisingly, in literature there is little consensus on the incubation times that have to be used for such digestions (Casadonte and Caprioli, 2011; Groseclose et al., 2007; Heijs et al., 2015; Sio et al., 2015). A workshop organized by the large European MSI network COST Action BM1104 that focused on comparing on-tissue digestion methods and data found little consensus and significant variation.

For example, in brain tissue white and grey matter are known to have distinct molecular compositions with different chemical characteristics, e.g. white matter is richer in lipid content making it more hydrophobic (Kaufman and Bard, 1999; Watson, 2012). However, it is unknown whether these differences have an effect on the on-tissue enzymatic digestion. Here we have investigated the brain region-specific efficiency of on-tissue digestion using MALDI-MSI. Using three groups of mouse brain tissue sections (N = 9) we varied the length of the incubation time: (i) 1.5 hours, (ii) 3 hours, and (iii) 18 hours. To get more quantitative insight into the success of the digestion, three previously identified proteolytic fragments originating from myelin basic protein (MBP, MBP_MOUSE, UniProt: P04370), were synthesized as isotopically-labeled reference standards (ILRS). Following the digestion incubation, the ILRS peptides were homogeneously sprayed on the tissue sections to enable intensity normalization of the endogenous MBP fragments. In this manner ionization bias could be traced throughout the tissue (Hamm et al., 2012; Källback et al., 2012; Porta et al., 2015) and throughout the evolution of the on-tissue digestion experiment.

Although the on-tissue digestion method is very effective and its application more commonly used, little has been reported about the repeatability, dynamics and spatial uniformity of on-tissue digestion in histologically heterogeneous tissue sections. One of the most comprehensive studies regarding on-tissue digestion, recently reported by Diehl *et al.* (Diehl et al., 2015), compared many parameters such as incubation time, type of MALDI matrix, and protease, but the main focus was to improve spatial resolution and image quality. The dynamics of the digestion, its repeatability and region-specific differences were not addressed. To the best of the author's knowledge, this is the first in depth investigation into the dynamics and mechanics of on-tissue digestion MALDI-MSI in a region-specific manner.

2.2 Methodology

2.2.1 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) except ethanol

(Merck, Darmstadt, Germany). Trypsin was purchased from Promega (Madison, USA). The isotopically-labeled peptide standards were produced by the Peptide Synthesis facility at Leiden University Medical Center (Leiden, Netherlands).

2.2.2 Isotopically labelled peptide standards

Isotopically labeled variants containing L-Threonine-$^{13}C_4$,^{15}N, (+5 Da, designated T*) of the following, previously identified (Heijs et al., 2015), tryptic peptides of myelin basic protein (MBP_MOUSE, UniProt P04370) were synthesized: (i) T*THYGSLPQK – *m/z* 1,136.5 Da, (ii) T*QDENPFFHFFK – *m/z* 1,465.71 Da, and (iii) T*QDENPFFHFFK^NIVTPR – *m/z* 2146.1 Da (missed cleavage, ^). The isotopically labeled peptides were dissolved in 0.1% trifluoroacetic acid (TFA) and mixed to get an equal response for each peptide from the mass spectrometer: (i) *m/z* 1,136.5 Da – 500 nM, (ii) *m/z* 1,465.71 Da – 100 nM, and (iii) *m/z* 2,146.1 Da – 75 nM.

2.2.3 Sample collection

A three-month-old, male, C57BL/6J mouse was sacrificed by cervical dislocation. The brain was excised, flash-frozen on dry ice and stored at -80°C until analysis. Twelve-µm thick coronal tissue sections (-1.06 – 0.74 mm bregma) were obtained using a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at -12°C. Three adjacent sections were thaw-mounted per poly-L-lysine coated indium-tin-oxide (ITO) glass slide (Bruker Daltonics, Bremen, Germany) and stored at -80°C. For every incubation time a group of three slides was prepared, resulting in 9 replicates for each incubation time (Figure 2-1A). All experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

2.2.4 Sample preparation

Tissue sections were brought to room temperature in a freeze-drier for 30 minutes. The tissue sections were then washed for 30 s in 70% ethanol, 30 s in 96% ethanol, 10 dips in deionized water, 30 s in 70% ethanol, and 30 s in 96% ethanol. Trypsin (20 ng/µL in deionized water) was then applied using a SunCollect automatic sprayer (SunChrom, Friedrichsdorf, Germany) as previously described (Heijs et al., 2015). In order to investigate the digestion dynamics different incubation times (1.5 h, 3 h & 18 h) were used. The digestion was incubated at 37°C in an airtight saturated chamber (50% methanol in deionized water). Proteolysis was terminated by covering the tissue sections with the mixture of isotopically labeled peptides (in 0.1% trifluoroacetic acid (TFA)) using the SunChrom SunCollect sprayer. A total of 2 layers was sprayed at 5 µL/min using the same sprayer settings as the trypsin application (Heijs et al., 2015). Finally, MALDI matrix (25 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% acetonitrile (ACN) and 0.1% TFA) was applied using the SunCollect sprayer as described (Heijs et al., 2015).

2.2.5 MALDI-FTICR imaging

MALDI-FTICR-MSI was performed using a 9.4 T SolariX XR mass spectrometer (Bruker

Daltonics) in positive-ion mode, using 150 laser shots per spot and 100 x 100 µm pixel size. Data was acquired for each tissue section and additional areas adjacent to the tissues to function as a matrix control. Spectra were recorded in the *m/z* range 600–3,500 Da with a 512k data point transient (1.1 s duration), corresponding to an estimated resolution of 200,000 at *m/z* 400 Da. The average detected mass resolution over the analyzed mass range was 54,000, and the mass resolution in the center of the mass range, at *m/z* 1,500, was 65,000. Data acquisition was performed using ftmsControl (Bruker Daltonics) and peptides visualized with flexImaging 4.0 (Bruker Daltonics). After MSI data acquisition the MALDI matrix was removed by washing the glass slides in 70% ethanol (2x 2 min), then Nissl stained and digitally scanned using a digital slide scanner (IntelliSite Pathology Ultra-Fast Scanner, Philips, Eindhoven, The Netherlands). The scanned histology images were co-registered to the MSI datasets in flexImaging.

2.2.6 Data analysis

The digestion dynamics were determined globally for the full tissue sections, and locally for specific histological features present in the mouse brain sections.

2.2.6.1 Global analysis – total average spectra

For the global analysis, the average spectra for each of the analyzed sections and their respective matrix controls, obtained from flexImaging, were exported as comma-separated values (CSV) and read into the freely available software tool mMass (http://www.mmass.org) (Strohalm et al., 2008). Mass spectral processing was performed using the following settings: (i) baseline subtraction – precision 15, relative offset 25, (ii) smoothing – Savitzky-Golay, width 0.05 *m/z*, 1 cycle, (iii) peak picking – S/N ≥ 5, relative intensity ≥ 0.6%, (iv) deisotoping – max. charge 2+, isotope mass tolerance 0.02 *m/z*, isotope intensity tolerance 50%, remove isotopes and unknowns, and (v) recalibration to a reference list containing the masses of the isotopically labeled peptide standards. Using mMass' peak list comparison tool, the peaks present in the matrix control were removed from the tissue peak list leaving only tissue specific peaks. The tissues specific peaks were exported to Excel 2010 (Microsoft). In Excel the total number of tissue specific peaks were grouped per digestion time point, and statistical comparison of the time-associated changes performed. A Kolmogorov-Smirnov test was applied to test the groups for normal distributions. Differences in distribution between the three groups were investigated using a Kruskal-Wallis test. If it was found that a group was distributed differently with statistical significance ($p \leq 0.05$), a Mann-Whitney test was used instead.

2.2.6.2 Global analysis – myelin basic protein

To investigate the digestion of MBP, the intensities of the three selected MBP peptides and their isotopically labeled variants were extracted from the average spectra of the tissues. In Excel, the intensities of the endogenous peptides were normalized using the intensities of the ILRS using (1).

$$I_{norm} = \frac{I_{endogenous}}{I_{ILRS}} \quad (1)$$

Statistical comparisons of the time-associated changes were performed similar to the analysis of the full spectra (detailed description above).

2.2.6.3 Local analysis – MBP digestion in white and grey matter

The alignment of the histology images to the MSI datasets facilitated the extraction of mass spectral data from specific histological regions. Two specific regions, equal in size (i.e. number of pixels) were selected from each tissue section: (i) white matter, a part of the lateral forebrain bundle system and, (ii) grey matter, a part of the cerebral cortex. The resulting XML files listed all spectra contained within these regions of interest (ROIs).

The MALDI-MSI datasets and corresponding XMLs were read into Data Analysis 4.2 (Bruker Daltonics), average spectra were calculated for all ROIs and peak picking was performed using the FTMS peak picking algorithm (S/N ≥ 5, Rel. Int. ≥ 0.6%). The intensities of the three MBP fragments and their respective isotopically labeled standards were exported to Excel 2010. In Excel the intensities of the endogenous proteolytic peptides were normalized to their respective ILRS, using (1). Statistical analysis of the histology-associated differences was performed as described above for the full average mass spectra.

2.3 Results and discussion

The on-tissue enzymatic digestion of proteins is used in MALDI-MSI to increase proteome coverage and assign protein identities to the peaks detected by MALDI-MSI. It is known from efforts to perform absolute protein quantification using LC-MS/MS that enzyme incubation time determines the completeness of the protein digestion and also the repeatability of the analysis (Brownridge and Beynon, 2011). Accordingly, we investigated the completeness of the digestion and the repeatability of on-tissue digestion by varying the incubation time and analyzing the resulting peptides using MALDI-MSI. A series of mouse brain tissue sections was analyzed: three groups of 9 tissue sections were digested for (i) 1.5 hours, (ii) 3 hours, or (iii) 18 hours and analyzed using a 9.4 T MALDI-FTICR instrument (Figure 2-1A).

2.3.1 Global analysis – total average spectra

The average mass spectrum from each complete tissue section was extracted, processed (background subtraction, smoothing), peak-picked and then recalibrated using the ILRS peptides as internal calibrants (Figure S2-1, Supplementary information). To obtain the number of tissue specific ions per tissue section, each tissue section's peak list was corrected by subtracting all peaks detected in the matrix control area adjacent to the tissue section. This correction removed all *m/z* features originating from the MALDI matrix, protease, and isotopically labeled reference

standard, leaving only tissue-specific *m/z* features. The resulting peak lists were deisotoped, grouped according to incubation time and then averaged (Figure 2-1B). A Mann-Whitney test was performed to calculate the statistical significance of the differences observed between the number of tissue-specific peptide ions per incubation time. It was found that after a 1.5-hour incubation the number of peptide ions was significantly lower, compared to the 3-hour and 18-hour incubations. No significant difference was observed between the number of tissue specific peptide ions from the 3-hour and 18-hour incubations. Although the number of peaks did not differ significantly between the 3-hour and 18-hour incubations, the variability was considerably larger in the 3-hour group compared to the 18-hour group. Great care should be employed when interpreting such peak lists of MSI data because the continuous production of new proteolytic fragments results in an increasingly complex chemical environment, which is known to cause ion suppression in MALDI mass spectrometry (Stauber et al., 2010).

2.3.2 Global analysis – myelin basic protein

To acquire more clarity into the dynamics of on-tissue digestion, a single protein was selected and isotopically labeled variants of the tryptic peptides were synthesized for use as internal reference standards. In this manner the ion suppression could be traced throughout the tissue and during the evolution of the digestion. Myelin basic protein (MBP, MBP_MOUSE, UniProt: P04370) was selected owing to its ready detection by MALDI-MSI (Clemis et al., 2012; Diehl et al., 2015; Heijs et al., 2015) and its distinct localization in the brain. MBP is an abundant protein in the central nervous system and has a crucial role in maintaining the multi-layered myelin membrane that builds up the myelin sheaths covering the nerve cells axons (Siegel et al., 1999). MBP is known to interact with the lipids in the myelin membrane and has a strong expression in regions consisting largely of white matter (Figure 2-2A-B) (Min et al., 2009; Siegel et al., 1999). Three of the MBP proteolytic fragments frequently reported by on-tissue digestion MALDI-MSI were selected: (i) TTHYGSLPQK (196-205, 1,131.5793 Da), (ii) TQDENPFFHFFK (211-222, 1,460.7168 Da), and (iii) TQDENPVVHFFKNIVTPR (211-228, 2,141.1138 Da). Fragment 211-228 is a missed-cleavage peptide that indicates incomplete digestion. Fragment 211-222 is the 'limit peptide' produced by the complete digestion of fragment 211-228, so any differences in their relative intensities may be interpreted as differences in the relative completion of the digestion. The isotopically labeled reference standards for the three MBP peptides were synthesized using a N^{15} and C^{13} labeled threonine residue ($\Delta[M+H+] = +5$ Da) incorporated on the N-terminal side of the three MBP fragments. A mixture of these peptides was prepared such that their intensities were similar to those of endogenous MBP fragments. From the respective concentrations for each of the three isotopically-labeled MBP peptides in the ILRS peptides mixture ((i) *m/z* 1,136.5 Da – 500 nM, (ii) *m/z* 1,465.71 Da – 100 nM, (iii) *m/z* 2,146,1 Da – 75 nM) it appears that the analysis of missed-cleavage peptides by MALDI mass spectrometry does not exhibit a similar bias in analysis sensitivity as

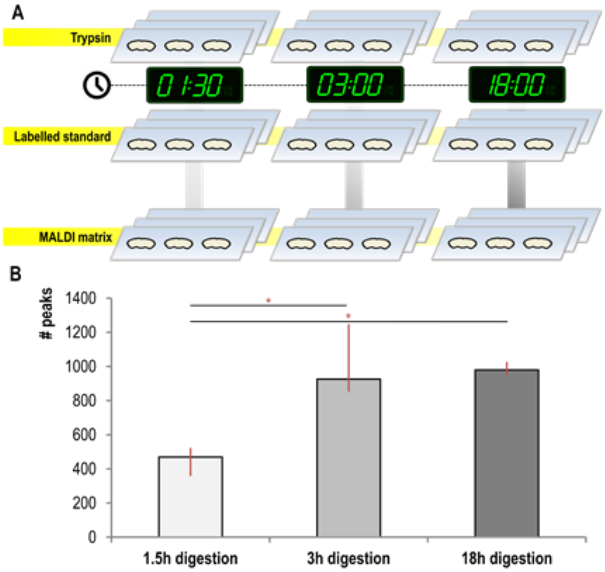

Figure 2-1: (A) Overview of the experiments. For each of the incubation times nine tissue sections were analysed. Three sections were mounted on one glass slide, the three slides were analysed on different days. **(B)** The number of tissue specific peaks (median) for each of the incubation times. The error bars represent the first and third quartile values. The asterisks represent $p \leq 0.05$.

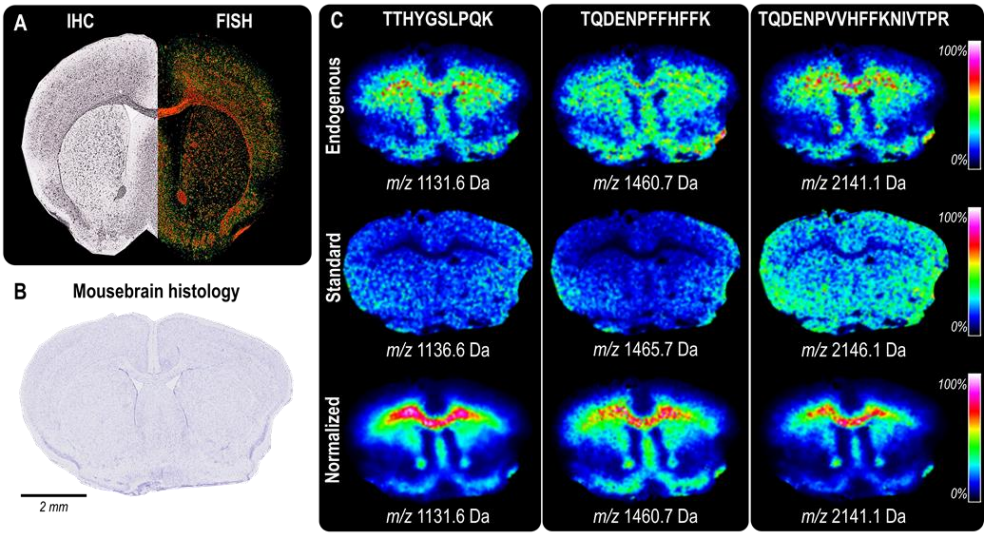

Figure 2-2: (A) Immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) data for myelin basic protein from the Allen Mouse Brain Atlas (©2015 Allen Institute for Brain Science. Available from: http://mouse.brain-map.org). **(B)** A scanned image of the histology (Nissl staining) of the MSI analyzed tissue section. **(C)** The effect of normalization to ILRS peptides on the distributions of MBP determined by on-tissue digestion MALDI-MSI. The top row contains the non-normalized images of the proteolytic fragments. The middle row shows the intensity distribution of the ILRS peptides, and the lower row shows the normalized images.

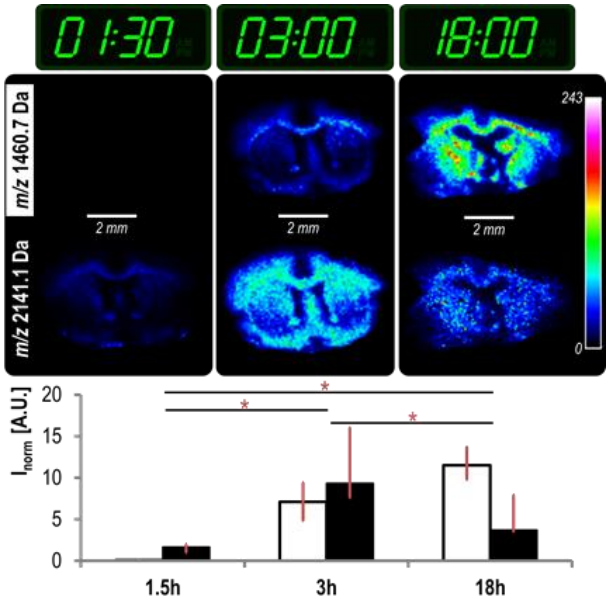

Figure 2-3: ILRS normalized visualizations of the MBP 'limit peptide' at *m/z* 1,460.7 Da (TQDENPFFHFFK) and missed-cleavage peptide *m/z* 2,141.1 Da (TQDENPFFHFFKNIVTPR) for each of the three incubation times. The bar plot shows the normalized intensities (median) for both the 'limit peptide' (white) and the missed-missed cleavage peptide (black) for each of the incubation times. The error bars represent the first and third quartile values and the asterisks p ≤ 0.05.

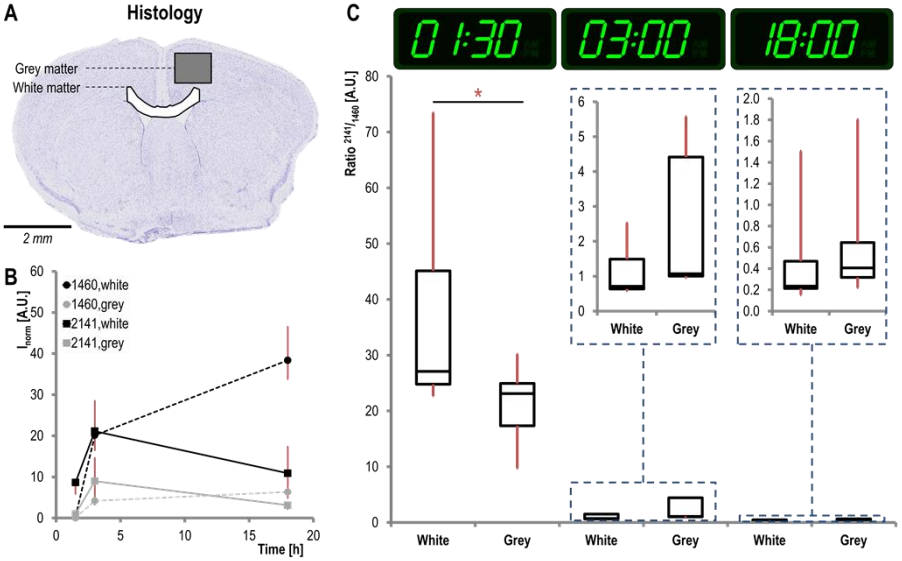

Figure 2-4: Comparison of MBP on-tissue digestion in mouse brain white and grey matter. **(A)** Selection of regions consisting largely of white and of grey matter that contain identical numbers of pixels. **(B)** The normalized intensities of 'limit peptide' *m/z* 1,460.5 Da and missed-cleavage peptide *m/z* 2,141.1 Da in regions with white and grey matter plotted versus incubation time. The error bars represent the 1st and 3rd quartile values. **(C)** Box plots showing the ratio of the intensity of the missed-cleavage peptide to the 'limit peptide' in white and grey matter over time. The insets show magnifications of the boxplots for 3 h and 18 h. The asterisks represent p ≤ 0.05.

during the analysis by electrospray ionization (ESI)-based mass spectrometry (Brownridge and Beynon, 2011). The equal sensitivity for the analysis of both 'limit peptides' and missed-cleavage peptides in MALDI-MSI emphasizes the need to drive the on-tissue digestion to completion, as the detection of missed-cleavage peptides will affect the reproducibility of the analysis.

Following the MALDI-MSI analysis the intensities of the endogenous peptides were normalized to the intensity of their ILRS peptides. Figure 2-2C shows the effect ILRS normalization has on the visualization of the peptide distributions. Visualization of the homogeneously applied ILRS peptides on the mouse brain tissue sections clearly shows that there is more suppression within the lateral forebrain bundle system, which is a part of the brain's white matter, as compared to the rest of the tissue section. The impact of ILRS normalization has been previously described for small molecules, such as pharmaceuticals and endogenous metabolites, and for proteolytic peptides in MALDI-TOF-MSI and MALDI-MRM-TOF-MSI (Clemis et al., 2012; Hamm et al., 2012; Prideaux and Stoeckli, 2012; Shariatgorji et al., 2014).

Following the normalization of the endogenous MBP peptides to their ILRS analogues the experiments with different incubation times, and the different regions could be quantitatively compared. The average (normalized) intensity was calculated for each incubation time and for each ROI to find whether the incubation time had a significant effect on the on-tissue digestion of MBP (Figure 2-3, Figure S2-2, Supplementary information). The presented data indicates significant differences between the intensity distributions of all three MBP fragments between 1.5 h and 3 h incubations. Additionally, the 211-222 (*m/z* 1,460.7 Da) and 211-228 (*m/z* 2,141.1 Da) fragments also show significant differences between the 3 h and 18 h incubations. As expected, the intensity distributions of both 'limit peptide' and missed-cleavage peptide have an inverse correlation, indicating that between the 3 h and 18 h time points MBP digestion is still ongoing.

2.3.2 Local analysis – MBP digestion in white and grey matter

It is known that the varying chemical composition of different organs/regions-of-tissue can have different mass spectral responses, leading to biased MSI representations of the true peptide distribution (Hamm et al., 2012; McDonnell and Heeren, 2007). Figure 2-2C shows such difference in ionization response for all three MBP peptides between regions in the mouse brain that contain white or grey matter. In this study we have investigated whether the different physical-chemical properties of the different morphological regions also affect the proteolytic digestion. Two regions, equal in size, from both grey and white matter were selected in each of the analyzed tissue sections and the spectra were extracted (Figure 2-4A). The ILRS normalized intensities of the MBP fragments 211-222 (*m/z* 1,460.7 Da) and 211-228 (*m/z* 2,141.1 Da) were determined for the two regions, and plotted versus time (Figure 2-4B). These results indicate a greater abundance of MBP in the region with white matter as compared to

the region with grey matter, which is in line with mRNA expression and immunohistochemistry results obtained from the Allen Brain Atlas (Figure 2-2A). Furthermore, it appears that MBP digestion is performed faster in white matter compared to grey matter as the equilibrium intensity between the 'limit peptide' and the missed-cleavage peptide lies close to 1.5 h in white matter and close to 12 h in grey matter. Furthermore, statistical analysis of the ratio between the missed-cleavage peptide and the 'limit peptide' (ratio = I_{2141}/I_{1460}) showed a significant difference between white and grey matter after only 1.5 h of incubation (Figure 2-4C). However, the results also indicated that these morphology-induced differences did not persist after 3-h and 18-h incubations, providing further evidence that a longer incubation provides more repeatable and reliable data.

In the current work we have demonstrated that in order to improve on-tissue digestion MALDI-MSI data longer incubation times are required. By performing an 18-h incubation, we were able to decrease the variability of the data sets regarding the number of tissue specific peaks. Furthermore, we were able to show differences in the digestion of MBP: (i) the missed-cleavage fragment 211-228 (*m/z* 2,141.1 Da) was significantly less abundant after 18 h compared to 3 h incubation time, (ii) the 'limit peptide' (fragment 211-222, *m/z* 1,460.5 Da) resulting from the digestion of the missed-cleavage peptide significantly increased after 18 h compared to 3 h. More importantly, for short digestions we were able to show differences in MBP digestion induced by the tissue morphology. These results urge caution owing to the numerous sources of technical bias in the MALDI-MSI data after short on-tissue digestion incubation times; as repeatability was improved, digestion more complete and morphology associated bias absent at longer incubation times, longer incubation times are advised.

2.4 Conclusions

Here we present the first in-depth investigation into the mechanics and spatio-temporal dynamics of on-tissue enzymatic digestion. To improve the repeatability and comparability of the MALDI-MSI data, normalization of targeted proteolytic fragments of MBP using isotopically-labeled reference standards was applied. The results indicate that a longer incubation of the enzymatic proteolysis reaction improves the quality of on-tissue digestion MALDI-MSI datasets by making the datasets more repeatable and remove morphology-induced measurement bias. This work is another step towards more trustworthy data obtained from on-tissue digestion MALDI-MSI analyses and towards the further implementation of the technique to clinical research.

2.5 Acknowledgements

This work was supported by the ZonMW Zenith project Imaging Mass Spectrometry-Based Molecular Histology: Differentiation and Characterization of Clinically Challenging

Soft Tissue Sarcomas (No. 93512002; BH) and Marie Curie IAPP Program BRAINPATH (No. 612360; AvdM & EAT).

2.6 Supplementary information

The full supplementary information are available on the internet via http://pubs.acs.org/doi/abs/10.1021/acs.jproteome.5b00849.

2.6.1 Supplementary figures

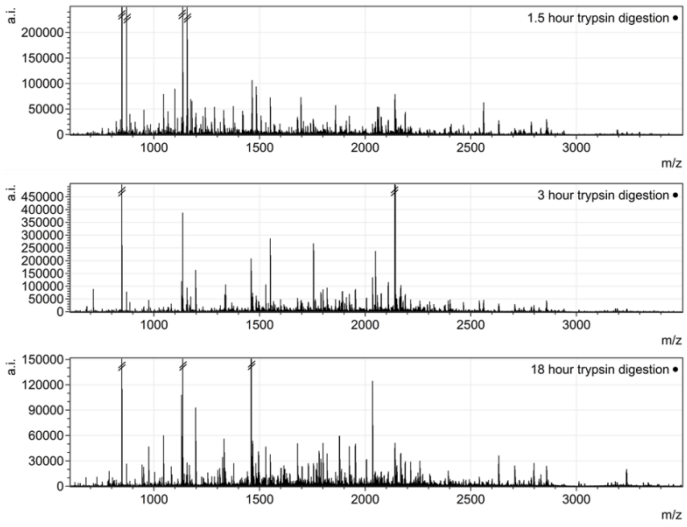

Figure S2-1: Examples of pre-processed average spectra for (top) 1.5 hour digestion, (middle) 3 hour digestion, and (bottom) 18 hour digestion.

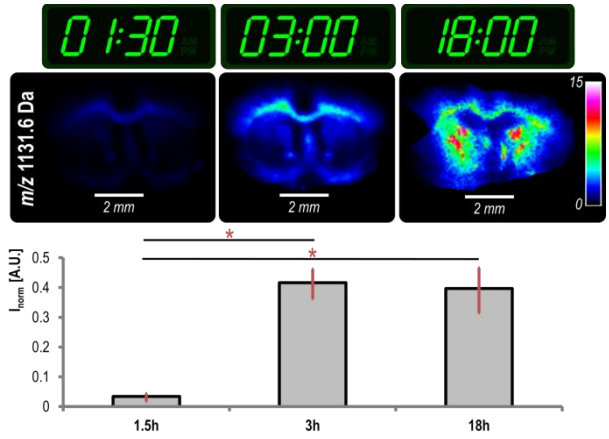

Figure S2-2: Visualizations of ILRS normalized 'limit peptide' at *m/z* 1,131.5 Da (TTHYGSLPQK) for each of the three incubation times. The plot shows the normalized intensities (median) for the 'limit peptide'. The error bars represent the first and third quartile values and the asterisks represents $p \leq 0.05$.