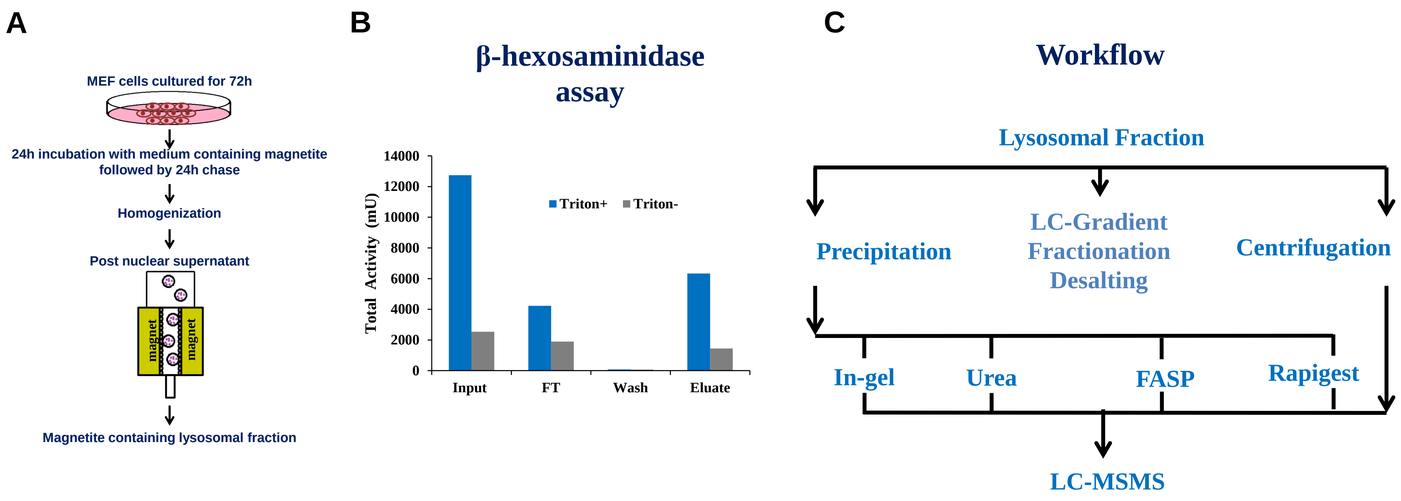
Optimization of sample processing for the analysis of lysosomal proteome

Srigayatri Ponnaiyan, Fatema Akter, Jasjot Singh, Dominic Winter
Institute for Biochemistry and Molecular Biology, University of Bonn, Germany
Email: s4srponn@uni-bonn.de

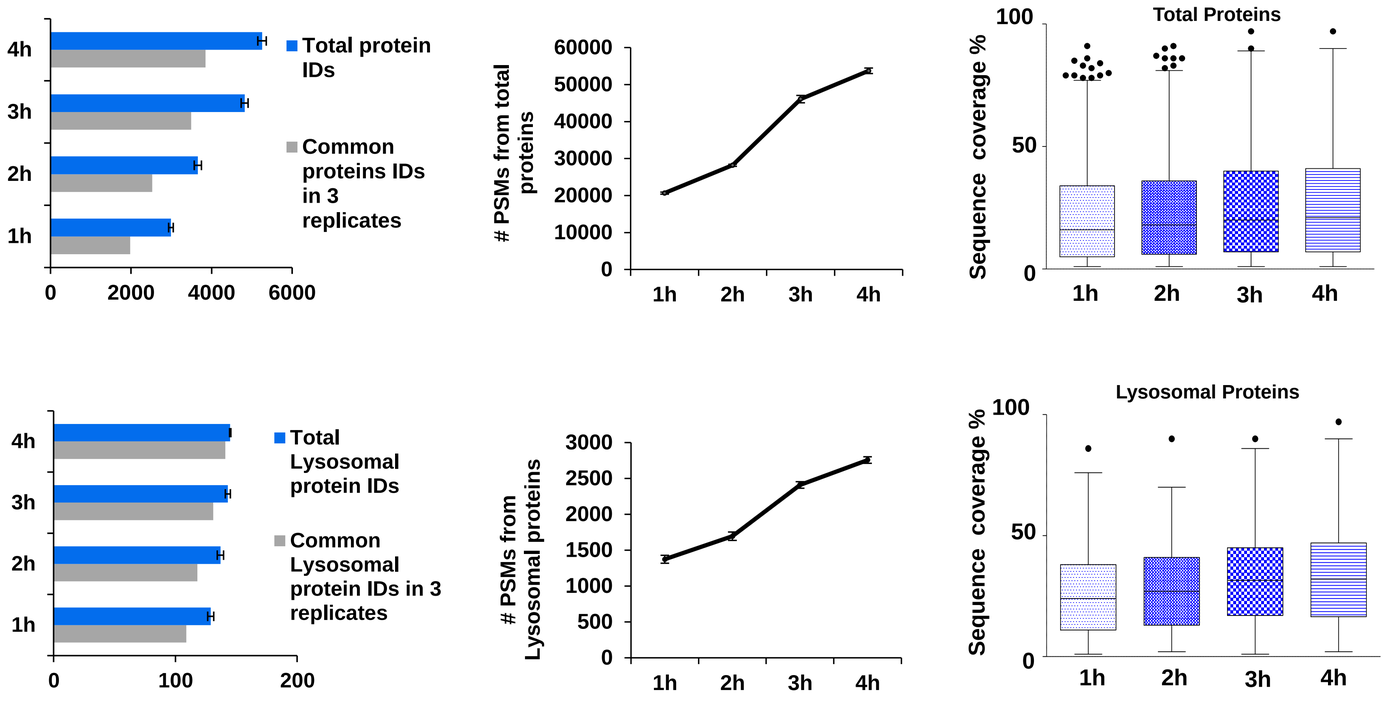
Background

Lysosomes, the main degradative organelles in eukaryotic cells, are composed of hydrolytic enzymes, proton pumps, ion channels, and heavily glycosylated membrane proteins playing roles in lysosomal structure, transport, signaling, and transport of molecules (1). Lysosomes are capable of degrading macromolecules such as proteins, nucleic acids, carbohydrates, and lipids, as well as whole organelles. In addition, lysosomes contribute to diverse cellular functions ranging from cell migration to metabolic signaling and gene regulation (2). Lysosomal dysfunctions are known to cause lysosomal storage disorders, and it is becoming more and more apparent that lysosomal dysfunction also contributes to common neurodegenerative diseases (3). Despite a growing interest in lysosomes, to date no systematic evaluation of protocols for the analysis of the lysosomal proteome by mass spectrometry based proteomics has been performed. In this study, we optimized the workflow for the analysis of the lysosomal proteome including proteolytic digestion, peptide fractionation, sample desalting, and LC-MSMS measurement.



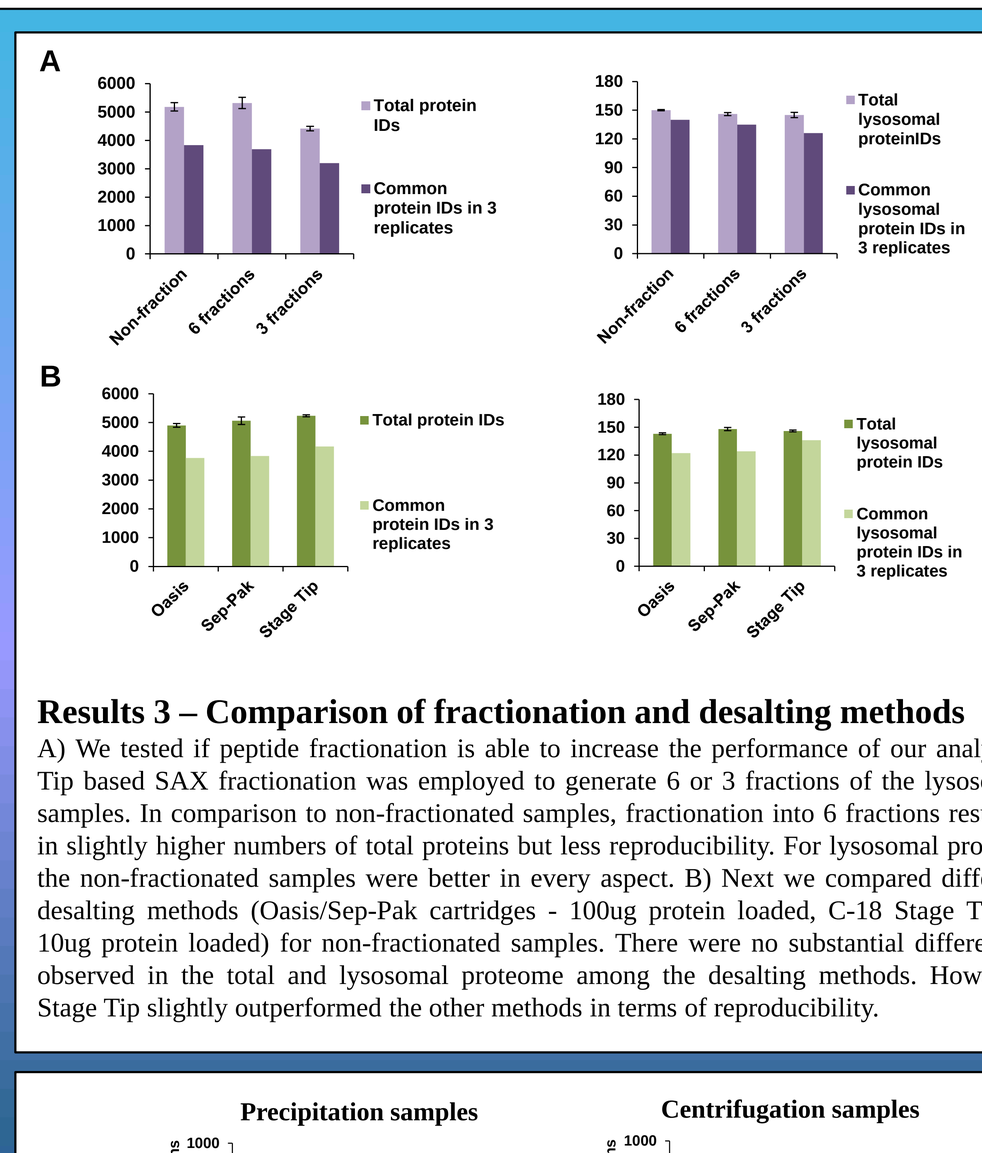
Results 1 – Enrichment of lysosomes

A) Lysosome-enriched fractions were obtained from mouse embryonic fibroblasts (MEFs) using superparamagnetic iron oxide nanoparticles (SPIONS). Magnetite (SPIONS), internalized by cells through endocytosis, were allowed to translocate to lysosomes during a chase period. After homogenization of cells, intact lysosomes were enriched by passing the post nuclear supernatant over a magnetic column. Lysosome enriched fractions were eluted after withdrawal of the magnetic field. **B)** The recovery and intact ratio of lysosomes in the enriched fractions was assessed using an enzymatic assay for the protein β-hexosaminidase which is present in the lysosomal lumen. Differences between ±Triton samples reflect the integrity of the lysosomes. We were able to recover 50% of the lysosomes present in the input 77% were intact. **C)** The workflow for the study is as follows: Lysosomal fractions were concentrated by 2 methods (i) precipitation of proteins by methanol:chloroform (PN) and (ii) centrifugation of lysosomes at 20,000g (CN). With both samples, we evaluated four proteolytic digestion methods, and peptide fractionation, sample desalting, and LC-MS gradient length for a representative sample. Samples were analyzed by LC-MSMS on an Orbitrap Fusion Lumos and the raw data were analyzed by Proteome Discoverer. All experiments were carried out in 3 independent replicates.



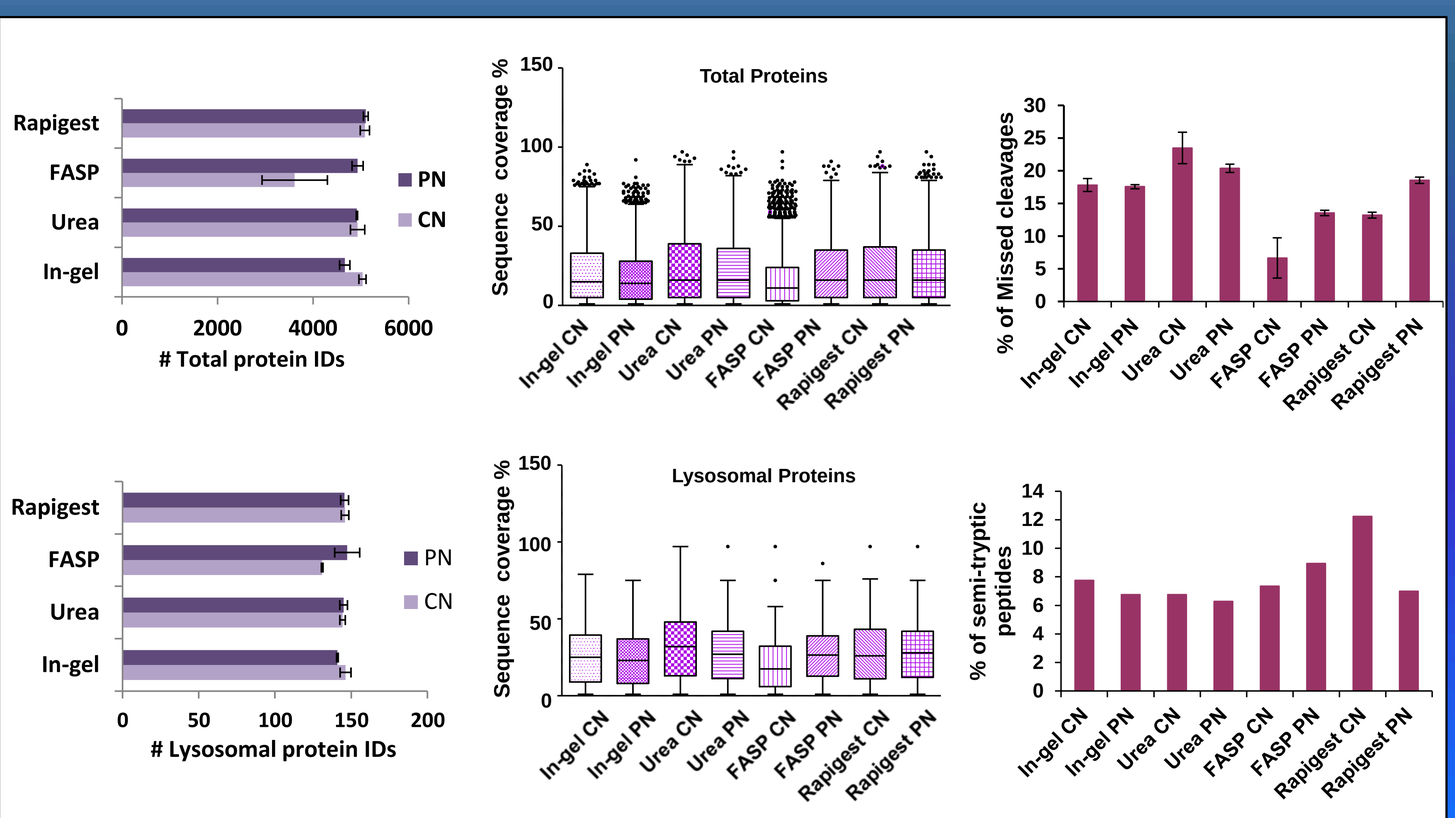
Results 2 – Comparison of gradient length

To determine the optimal gradient length required for measuring lysosome samples, we compared different gradient lengths of 1h, 2h, 3h, and 4h for unfractionated tryptic digested lysosome samples. Samples were searched against Uniprot and results matched against a shortlist of lysosomal proteins. For total as well as lysosomal protein IDs, the 4h gradient yielded best results while it was most pronounced when taking reproducibility of identification into account. We observed the same trend for PSMS. Also for sequence coverage 4h gradients performed best which was more pronounced for lysosomal proteins .



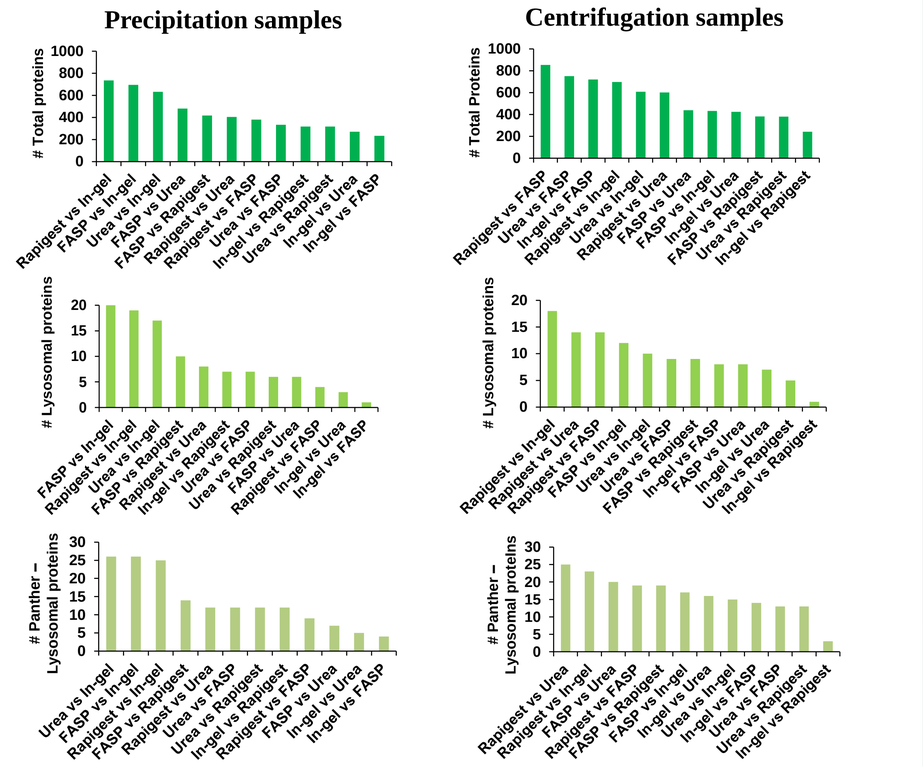
Results 3 – Comparison of fractionation and desalting methods

A) We tested if peptide fractionation is able to increase the performance of our analyses. Tip based SAX fractionation was employed to generate 6 or 3 fractions of the lysosomal samples. In comparison to non-fractionated samples, fractionation into 6 fractions resulted in slightly higher numbers of total proteins but less reproducibility. For lysosomal proteins the non-fractionated samples were better in every aspect. B) Next we compared different desalting methods (Oasis/Sep-Pak cartridges - 100ug protein loaded, C-18 Stage Tips - 10ug protein loaded) for non-fractionated samples. There were no substantial differences observed in the total and lysosomal proteome among the desalting methods. However, Stage Tip slightly outperformed the other methods in terms of reproducibility.



Results 4 – Comparison of digestion methods

Lysosomal fractions concentrated by methanol:chloroform precipitation (PN) as well as centrifugation at 20,000g (CN) were subjected to different digestion methods (in-gel, in-solution by urea and Rapigest, and FASP). The digested samples were desalted by C18- Stage Tips and 1µg of sample was used for MS measurement (4h gradient). All digestion methods resulted in virtually similar numbers of IDs (Proteins, PSMs, and unique peptides), except FASP which resulted in lower IDs for the CN samples. Even though urea exhibited the highest sequence coverage for the total and lysosomal proteome, it resulted in highest number of missed cleavage sites. On the other hand, Rapigest CN yielded the highest percentage of semi-tryptic peptides.



Results 5 – Label free quantification

Label free quantification was performed to identify the magnitude of protein abundance changes between each digestion method. Proteins which met the threshold of log2 > 1 with a p-value <0.05 were considered. Abundance values were analyzed for both total as well as lysosomal proteins. The lysosomal IDs assessed were from a list of lysosomal GO-terms (http://pantherdb.org) as well as a manually curated list based on the literature (4). Rapigest identified more protein IDs with a higher intensity in comparison to other methods while in-gel digested samples resulted in the lowest intensities observed.

Conclusion

Our data suggest that in solution digestion by both urea and Rapigest provide superior results for the analysis of the lysosomal proteome in comparison to in-gel and FASP. Desalting by C-18 Stage tips and analysis with a 4h gradient obtained maximal numbers of lysosomal protein identifications with highest reproducibility. Concentration by precipitation (PN) and centrifugation (CN) performed roughly similar for Rapigest and urea, while we observed differences for FASP and in gel digests. However, the CN samples digested with Rapigest showed a higher amount of semitryptic peptides which may be due to active lysosomal proteases during tryptic digestion.

References

1. Ballabio, A., The awesome lysosome. *EMBO molecular medicine* 2016, *8* (2), 73-76. **2.** Lim, C.-Y.; Zoncu, R., The lysosome as a command-and-control center for cellular metabolism. *J Cell Biol* 2016, *214* (6), 653-664. **3**. Ballabio, A.; Gieselmann, V., Lysosomal disorders: from storage to cellular damage. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 2009, 1793 (4), 684-696. **4.** Thelen, M., Winter, D., Braulke, T., & Gieselmann, V. (2017). SILAC-based comparative proteomic analysis of lysosomes from mammalian cells using LC-MS/MS. *Lysosomes: Methods and Protocols*, 1-18.