Development of a robust and reproducible method for detection of citrullination in complex samples

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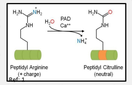
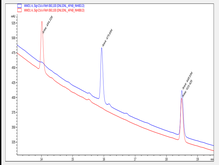
Introduction

Citrulline is a deamination of arginine, resulting in the exchange of NH to double bonded O, giving rise to a charge difference, mass shift of 0.985 Da, and thus resulting in different epitope on the protein/peptide. [4]



Although identification of citrulline is simple and can be done by all standard search engines, each spectrum has to be manually validated due to the possibility of wrong isotope picking, deamidation, and loss of charge. In order to simplify validation we developed a search tool, *Citrullia*, which integrates the X! Tandem search engine with extraction of potential citrullination, visualization of spectra, and MS1 spectrum information.

We have observed that different ion-pairing reagents results in different retention time shifts of citrullinated peptides and in the present report we show that is can be used for increased confidence in identification. Here we aim to develop a system where specific ion pairing reagents are used to separate arginine containing peptides from equivalent citrullinated ones. An initial separation by traditional LC using HFBA as ion-pairing reagent followed by short LC-MSMS separation on an EVOSEP system is compared to a traditional LC-MS/MS setup.

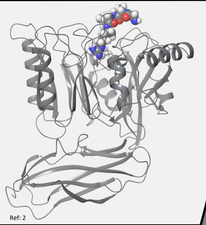


Blue graph = Non-citrullinated
Red graph = Completely-citrullinated

Only a few enzymes can convert an arginine into a citrulline. They are known as Peptidyl arginine deiminases. Five types are found in the human body and a single type is found in *P. gingivalis* [5]. With low to no sequence similarity or citrullination motif known, their similar function must be investigated to elucidate their function and possible interplay in disease onset and development.
A connection between Rheumatoid Arthritis (RA) and Periodontal Disease (PD) has been identified, where Anti-Citrullinated Peptide Antibodies (ACPA) have been found in both patients with RA and in patients with both Rheumatoid Arthritis and Periodontal Disease. *P. gingivalis* is present in high levels in PD patients together with ACPA's, indicating a connection and the possible citrullination of similar or identical epitopes. [3]

Porphyromonas gingivalis Peptidyl Arginine Deiminase (PPAD)

- Secreted from the periodontal pathogen *Porphyromonas gingivalis*
- 61.7 kDa
- Citrullinates arginines (deamination), with low specificity
  - Primarily C-terminal arginines,
  - Citrullination of internal arginines have also been observed
- Active at neutral to basic pH
- Activity is depended on L-cysteine
- No sequence similarity with the human PADs, but similar reaction type (arginine into citrulline)



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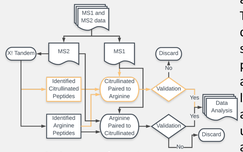
Citrullia – Program for validation

Problems in validation of citrullination

- Wrong isotope picking
- Bad fragmentation due to low charge or single charged peptides
- False negatives due deamidation of N or Q
- Discarded due to C-terminal location
- MS1 data not included in validation

Flowchart representing the data handling



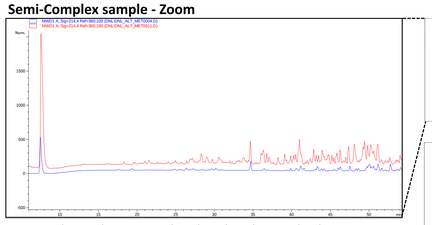
Validation/optimization

Data is loading using MGX file format, containing both MS1 and MS2 spectra. The MS2 data is searched using X! Tandem. MS1 data is used for quantification. Identified citrullinated peptides are matched to peptides with similar sequence, except having 0.984 Da higher parent ion mass. Peptides containing arginines are matched with spectra having 0.984 Da lower parent ion mass. The matches are analyzed and validated as pairs. The unpaired identified citrullinations are validated on their own and characterized as lonely.

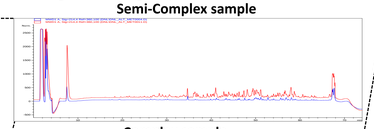
HPLC – Fractionation using HFBA

For optimal separation of citrullinated and non-citrullinated peptides, the hydrophobic ion pairing reagent heptafluoride butyric acid (HFBA) was used. The change in hydrophobicity shown with HFBA, resulted in earlier elution of citrullinated peptides compared to non-citrullinated peptides. The combination of a long gradient and small fractions (one each minute), should result in fraction separation of at least one fraction. High background was observed in all runs, but the addition and mix of HFBA with the sample prior to injection gave better result, illustrated in the Semi-Complex sample graphs. Furthermore, the complex sample graphs show very high background, indicating that optimization is necessary. Even though the background was high, separation was achieved. 48 fractions were collected in the initial experiment in one minute fractions from 6 min until 54 min. Further analysis was performed using an EVOSEP system with a 21 min gradient in Formic Acid and tandem MS. For optimal identification and validation complexity of the sample, gradient length, and retention time change between peptides containing arginine or citrulline must be taken into account.

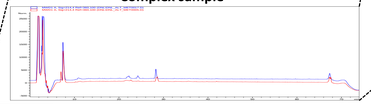
Semi-Complex sample - Zoom



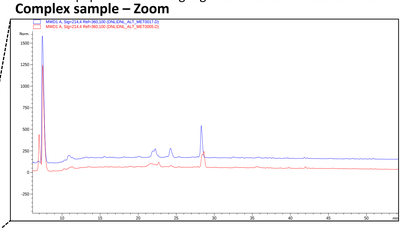
Semi-Complex sample



Complex sample



Complex sample – Zoom



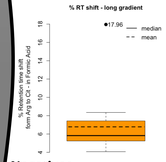
Blue graph = no mix with acid ; Red graph = mixed with HFBA

Blue and red graphs are replicates of Outer Membrane Vesicles from W83

Single run – 90 min gradient Versus 48 runs – 21 min gradient

Positives

- Extensively tested method
- More citrullinations



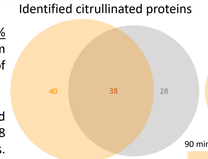
Negatives

- Low potential gain by optimization of method
- Few paired citrullinations

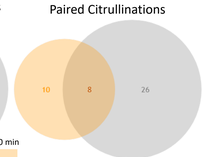
Retention time shift – long gradient
Average retention time shift of 5.82% have been observed, with a minimum shift of 4.07% and a maximum shift of 17.96%.

Comparison
Out of the 78 and 66 identified proteins containing a citrullination, 38 were common for the two methods. Furthermore, 43 citrullinated peptides were common, but unique citrullinations were obtained by either method. The 21 min, 48 run method, did identify more paired and thereby validated than the 90 min, single run method. Still unique citrullinations were found. Most of the un-paired citrullinations were found in the 90 min, single run method

Identified citrullinated proteins

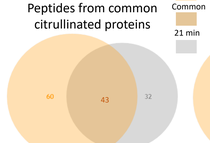


Paired Citrullinations

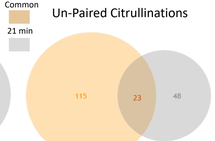


90 min
Common
21 min

Peptides from common citrullinated proteins

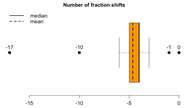


Un-Paired Citrullinations



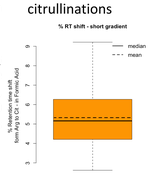
Retention time shift – short gradient
Average retention time shift of 5.16% have been observed, with a minimum shift of 2.62% and a maximum shift of 9.21%.

Fraction shift
Average fraction shift of 4.5 fractions have been observed, where citrullinated peptides elute earlier, with a minimum of 0 fractions (0 min) and a maximum of 17 fractions (17 min).



Positives

- Improvable method
- More paired citrullinations



Negatives

- Needs optimization
- More instrument time due to many fractions
- Fewer total citrullinations

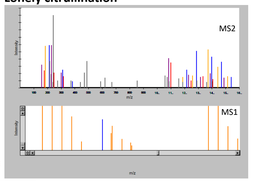
Validation of Citrullination

One sample – One run

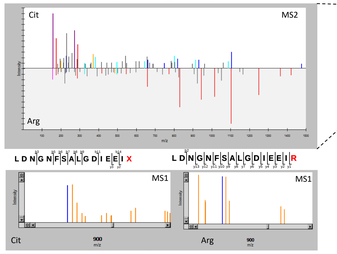
Validation parameters for single runs

- Minimum 2 y-ions (y1 + y2) + 3 b-ions
- Correct Isotope picking (If MS1 available)
- Retention time shift (Longer retention)

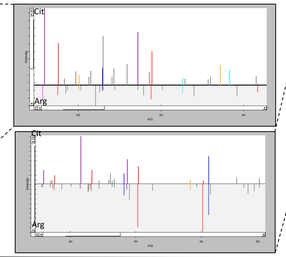
Lonely citrullination



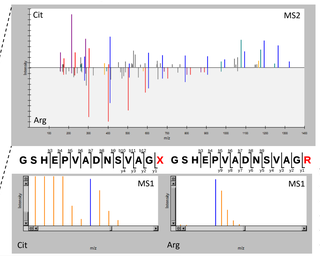
Citrullination with paired Arginine spectrum



Single Dalton Shift



One sample – 48 fractions – 48 runs



Characteristics

- y1-ion
- y1-(-17)-ion
- Many b-ions
- Few large y-ions
- RT shift in HFBA should elute earlier
- RT shift in Formic Acid should elute later

Validation parameters for 48 runs

- Minimum 2 y-ions (y1 + y2)
- Minimum 3 b-ions
- Correct Isotope picking
- Earlier RT in HFBA for citrullination
- Longer RT in formic acid for citrullination

Material and Methods

HPLC
Agilent 1260
Column - Phenomenex Aeris PEPTIDE 2.6 u XB – C18

Gradient
Flow = 200 µl/min ; Max pressure = 200 bar

Min	% B
0-2	5
2-62	5-45
62-63	45-90
63-65	90
65-65.3	90-5
65.3-74	5

RP-LC/MS/MS analysis
Traditional method

- C18 2-columns setup connected to an EASY-nanoLC 1000 system (Thermo)
  (90 min gradient – 120 min cycle time)
- Orbitrap Fusion Lumos Tribrid (Thermo)

Alternative method

- EVOSEP – C18 column and EVO-TIPS
  (21 min gradient – 24 min cycle time)
- Orbitrap Fusion Lumos Tribrid (Thermo)

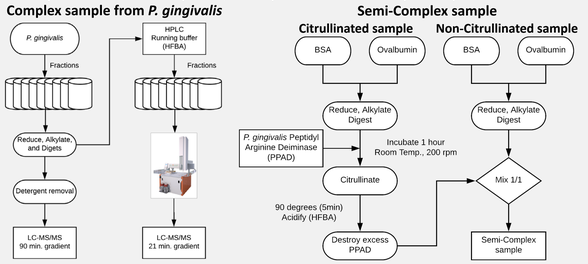
Data analysis

- Citrullia, Christian E. Mikkelsen
- GPMAW, Lighthouse data, Denmark; gpmaw.com
- Jol, S.J. (2015) *Make a Venn Diagram*. https://www.stefanjol.nl/venny

Sample Preparation

Two sample sets were prepared – Complex and Semi-Complex

A sample set was prepared using a mixture of BSA and Ovalbumin to illustrate the use in a simple sample set. Another set was prepared using various cellular fractions from *P. gingivalis*, (Jan Potempa Group), for illustrating a complex sample. The analysis using mass spectrometry with either a long gradient, 90 min, or a short gradient, 21 min, were performed for both sample sets, but only illustrated for the complex sample.



Conclusion

- **Citrullinated peptides elute earlier using HFBA on HPLC**
- **Citrullinated peptides elute later using FA on nano-LC**
  - Manual validation of MS1 and MS2 spectra, in addition to retention time shift, is necessary for proper validation of citrullinations in peptides
    - C-terminal citrullines were primarily observed

References

1. The Protein Experts. Cytoskeleton news. *Cytoskelet News*. 2012;3(Cc):19-20. doi:10.3389/fncel.2014.00314.Boulter
2. Montgomery AB, Kopec J, Shrestha L, et al. Crystal structure of Porphyromonas gingivalis peptidylarginine deiminase: Implications for autoimmunity in rheumatoid arthritis. Ann Rheum Dis. 2016;75(6):1255-1261. doi:10.1136/annrheumdis-2015-207656
3. Potempa, Jan, Piotr Mydel, and Joanna Koziel. "The case for periodontitis in the pathogenesis of rheumatoid arthritis." Nature reviews. Rheumatology (2017).
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5. Goulas, Theodoros, et al. "Structure and mechanism of a bacterial host-protein citrullinating virulence factor, Porphyromonas gingivalis peptidylarginine deiminase." Scientific reports 5 (2015): 11969.