Ultrasonic treatment in proteomic studies: direct processing of biopsy samples for subtyping of amyloidosis.

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Introduction:

In conventional bottom-up proteomics studies, proteolytic digestion is a decisive step of the analysis process. Ultrasonic (US) or microwave technologies have been used in enzymatic digestion [1,2]. In ultrasonic process, amount of energy and efficiency brought by cavitation effect, can significantly improve protein quality digestion and reduce preparation time.

Objectives:

Here we show that ultrasonic treatment could help for the completion of enzymatic proteolysis on raw or fixed amyloid biopsy samples (a disease where insoluble deposits of specific proteins occur in tissues) [3]. Diagnosis of different amyloidosis' classes mostly relies on histochemical approaches but can be inconclusive on certain cases. Subtyping has been recently demonstrated combining laser micro-dissection (LCM) and mass spectrometry [4].

Material & Methods

Consumables & operating parameters

•trypsin (bovin, porcin), modified or not

•Polypropylene tubes purchase from different suppliers (Treff lab, Epp.Lobind, Axygen): 3 references

•Standard protein used during tests and optimization, BSA (sigma A3059).

•Concentration solutions used in sensitivity tests: 1 pmol/µl, 100 fmol/µl et 10 fmol/µl in triplicate)

•MALDI TOF/TOF analysis: Spectra in positive mode

•Matrix CHCA 4mg/ml (MALDI-TOF/TOF 4800 ABSciex)

Biological Material & Preparation

Clinical Biological Material: Raw or fixed tissues (paraformaldehyde-immobilized / coloration Bouin or AFA) from patients or control (tissues declared healthy for this pathology).

Preparation: Direct proteolysis using ultrasonic process.

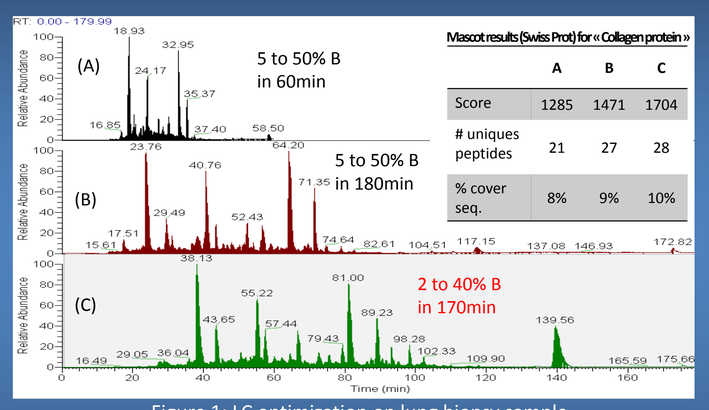
• tissue section (10µm): calculated volume extrapolated from anatomopathology section tissues.

• tryptic digestion : sonication (MicroSon XL probe - 4*C) after reduction-alkylation

• sample : filtration on Proxeon stage tips and reconstitution in same volume prior injection (roughly 0.15 mm3 were injected for each LC (except for the comparison with Laser Capture Microdisection sample where 0.0055 mm3 were injected , 0.3 equivalent slice)

System optimization & Analysis

Nano chromatographic system optimization on tryptic protein standard mixture then using biopsies (systems and gradients)



Mascot results (Swiss Prot) for « Collagen protein »

	A	B	C
Score	1285	1471	1704
# uniques peptides	21	27	28
% cover seq.	8%	9%	10%

Figure 1: LC optimization on lung biopsy sample

• nano C18 Acclaim pepMap100 (Dionex) Viper 75µm d.i.x15cm, gradient 2% to 40%B in 170 min, buffer: A H2O/ACN/AF 98:2:0.1 (v/v/v) / B H2O/ACN/AF 10:90:0.1 (v/v/v)

•Nano ESI source (Triversa Nanomate, Advion)

• LTQ-FTultra: FTMS Resolution 50000 MS + top 7 CID LIT MS/MS (Thermofisher scientific)

•Mascot 2.4 and Sequest on Proteome Discoverer 1.3.0.339

•UniProt-SwissProt 2013 and home made SwissProt/Trembl database containing amyloidosis proteins.

•MS 5ppm, MSMS 0.5Da, up to 2 miss cleavages, full tryptic peptides, Ox (M) and CAM (C) as partial modifications.

•Validation criteria: only consensus proteins identified in triplicate with both search engine with a FDR<1% are kept.

Results

Enzymatic stability Study : Four different trypsin are experimented to compare ultrasonic process to conventional digestion (TX: manual, thermomixer 2h30, 37°C) measuring relative intensity of 3 major peptides of BSA digest (5fmol/ul).

=> **Modified bovin trypsin is rejected for US since only few tryptic peptides are found in MALDI TOF spectra (not shown).**

Consumables behaviour during ultrasonic treatment, digestion efficiency and low retention for digest product

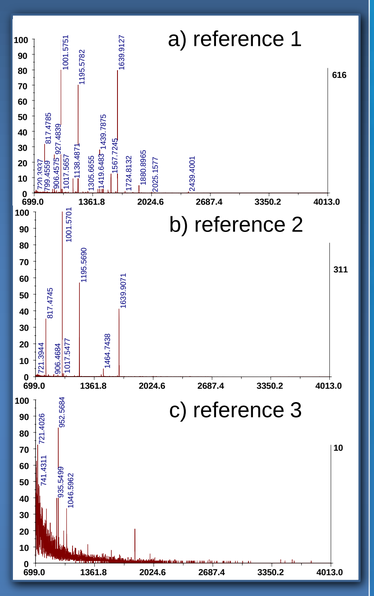


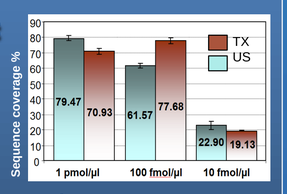
Fig 2: Digest results for 3 references tubes (BSA 25fmol/µl, digest vol. 100ul)

•Only reference 1 (fig 2a) leads to similar relative intensity spectra and detected number of peptides than conventional process (TX).

=> Consumable choice must considered composition and surface treatment to minimized sample loss.

Sequence coverage and sensitivity:

Fig 3 : Sequence coverage (%) with US assisted and conventional digestion, for different BSA concentrations



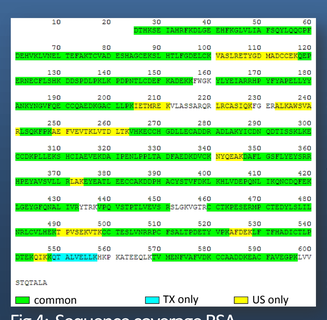


Fig 4: Sequence coverage BSA

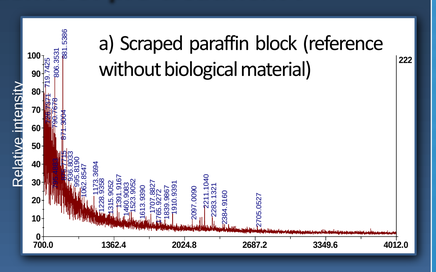
•On low sample concentrations sequence coverage (fig 3) is better with US. Incomplete digestion leads to miss cleavage (fig4) that allows detecting part of sequence represented by smaller peptides.

=>New peptides species increasing coverage with US.

Clinical biological material: Influence of pre-treatment :

Paraffin : Only tryptic peptides and usual contaminants. No polymers detected (fig 5a).

=> No interferences from paraffin for mass spectrometric analysis.



Formalin fixed tissues:

•Paraffin or deparaffinized scraped slices produce high quality spectra (fig5b) that allows for **proteins identifications** (Tab 1).

protein identification NCBI	Acc.Number	Prot. score	Detected in
immunoglobulin kappa, VJ region	gi|1322204	99	b1
Ig kappa chain VKIII-JK5	gi|470430	70	b1
immunoglobulin gamma-chain, V region	gi|567160	62	b1
immunoglobulin kappa light chain variable region	gi|4378326	61	b1 / b2
immunoglobulin light chain variable region YV1-14-K3-6	gi|84798278	59	b1 / b2

Tab 1: MALDI/TOF identifications for scraped deparaffinized and paraffin embedded sample.

Paraffin removal step :

•Relative intensity on paraffin sample spectra (fig 5c) is higher than deparaffinized sample (fig 5d)

=>Possibility of direct analysis of formalin fixed paraffin embedded tissues by MALDI/MS. No need to add another step prior US.

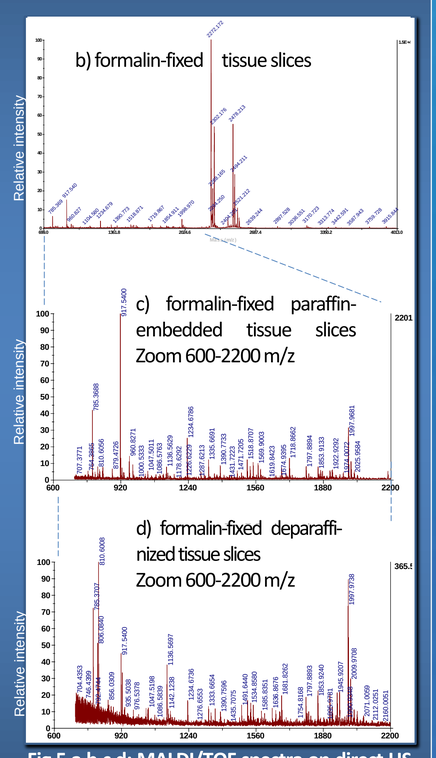


Fig 5 a,b,c,d: MALDI/TOF spectra on direct US proteolysis sample.

Raw material vs fixed tissues :

•Ultrasonic tryptic treatment is performed on raw or fixed tissue slices collected (lung, kidney, testis, salivary glands (ASGB), spleen…) and fixation tissues processing (Bouin/AFA) (data not shown).

=>No impact of origin/preparation tissues for subtyping of amyloidosis is detected.

Subtyping amyloidosis: discovery approach.

•**Proteolytic** peptide mixtures are analyzed by nanoLC-MS/MS.

•**Data interpretation**. Thirty six proteins associated to amyloidosis are considered in this study. Only one isoform is listed for each group, and only proteins validated and annotated in SwissProt, are considered. Abundances of the protein are evaluated according to (5) taking into account the area of the three most intense peptides.

•Unidentified species are given the minimum area that could be detected. Only biopsies exhibiting a relative abundance >1% and containing **Apolipoprotein E and Serum amyloid P component** (common to all amyloid deposits) are considered as potential amyloid candidate. Finally amyloidosis are classified according to the relative intensity of the 36 proteins.

Results: Data for various tissues are compared with **immunohistopathology** results using antibodies for amyloidosis diagnosis. One significant examples from ASGB with 2 pathologies illustrate our results.

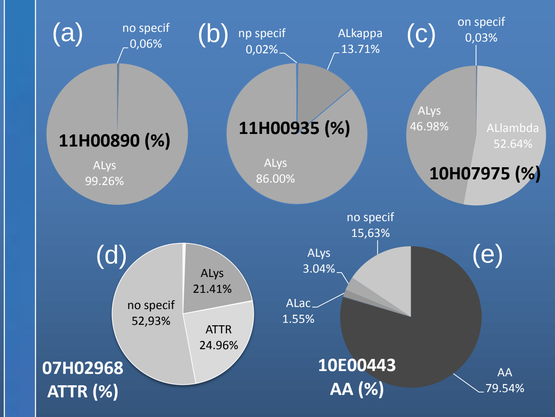


Fig6 a,b,c,d,e: The results are visualized with sector diagrams representing the relative intensities of amyloid proteins.

At the top (a,b,c): Biopsies of 3 negative controls. ASGB are enriched in lysozyme, no SAMP or ApoE is present.

At the bottom (d,e): Biopsies of amyloid (d) ATTR, (e) AA,

=> For biopsies in Fig 6 , the results are negative for ALκ and ALλ, and positive for (d) TTR and (e) AA respectively, using both techniques. ApoE and SAMP were also detected unambiguously among the major compounds (fig 6 d,e).

=> Ultrasonic tryptic treatment allowed performing subtyping of amyloidosis

Conclusions & Perspectives:

Ultrasonic treatment greatly minimized preparation time (from treatment to acquisition, less than two days are necessary) and reduced sample quantity. Combined with LC-MS/MS, it opens the way for an accurate amyloidosis diagnosis and subtyping directly from clinical samples and allows most of the time avoiding the LCM step (which could be time-consuming). This is of particular interest for classes that could not be distinguished by the classical histochemical analysis or identifying new amyloidosis.

Our next step will be to expand and validate our approach to different amyloidoses and tissues with clinicians. In order to increase cohorts for a realistic clinical application we intend to automatize the first step. Miniaturization is required to decrease sample consumption of the first treatment (only 10% of the sample is actually analyzed).

Specific approaches targeting isoforms interest are currently under analysis with parallel reaction monitoring (PRM / QExactive, Thermofisher scientific) and Selected reaction monitoring (SRM / TSQ Vantage, Thermofisher scientific) experiments **(POSTER P273 session2).**

References

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