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Which step to use between Streptavidin-biotin purification of proteins complexes and LC MSMS analysis?

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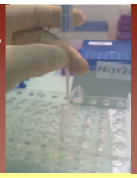
In order to identify the molecular partners of a protein of interest, widely used protocols consist of 1) biotin labeling of the protein of interest, 2)in vitro or in vivo incubation with candidate proteins, and 3)affinity purification of the complex. Then, proteins are eluted and, after enzymatic digestion, they are submitted to mass spectrometry analysis.

The **elution step** is of a crucial importance in this workflow. This step is critical because the biotin-streptavidin complex is very stable ($K_D = 10^{-15}$ M). An ideal elution should allow releasing of all the proteins in a buffer and a volume compatibles with enzymatic digestion and proteomic analysis.

Another possible way could be to perform the enzymatic digestion directly on the beads, and to elute the peptides.



Aim of the work

This works aims to evaluate, on a complex biological sample, several procedure allowing the identification of the molecular partners of a biotinated protein purified with streptavidin-coated beads.
In particular, a protocol usin g on-bead trypsic digestion was used.
The biological significance of the identified proteins was assessed with a functional assay.

MATERIEL & METHODS

Biological material

- Control: 2 x 10^8 1F3 drosophila cells
- Sample: 2 x 10^8 1F3 drosophila cells transfected with the Dredd protein (involved in the immune anti-bacterial response) , N-terminus tagged with biotin

- 10 mn after a bacterial challenge (to activate the Dredd complex), control ans sample cells lysis
- affinity purifcation of the complex with Streptavidin coated on sepharose beads
- final volume: 200 µL, in 4 tubes of 50 µL.

Protein elution:

After 2 washing, proteins are eluted with one of the following protocols

Name	Elution Buffer	Incubation	Ref
Ethanol	30% Ethanol, 1% TFA Vf = 50 µL	30 mn at RT	[1]
Glycin	0.1 M Glycin pH 2.5 Vf = 50 µL	10 mn at RT Neutralization 1M Tris pH 8.8	[2]
Biotin	30 mM biotin, 2% SDS, 160 mM NaCl, 6M urea, 2 M thiourea. Vf = 50 µL	15 mn at RT and 15 mn at 96 °C	[3]
Laemli	Laemli buffer, Vf = 50 µL	10 mn at 96°C	

Half of the eluted proteins are submited on liquid trypsic digestion, and the other half is fractionated on SDS-PAGE gel before digestion

On bead digestion (before elution)

After reduction/alkylation (DTT 5mM final, 30 mn, 56°C / Iodoacétamide 25mM final 20 mn in dark),
incubation overnight with10 ng trypsin (Promega).
The peptides are recovered and the beads eliminated by using C18 Proxeaon tips and 50% methanol – 5% FA (see photo) in Vf = 60 µL

Liquid digestion (after élution)

For protocol « Biotin », SDS is first removed by using ZipTip $_{HPL}$ column (Millipore).
For all protocols, reduction/alkylation (DTT 5mM final, 30 mn, 56°C / Iodoacétamide 25mM final 20 mn in dark),
incubation overnight with10 ng trypsin (Promega).
Buffer are exchanged against 20 µL buffer A.

In gel digestion

12% poly-acrylamide gel stained with Coomassie blue. All the gel is cut (not only stained band) reduction/alkylation (DTT 5mM final, 30 mn, 56°C / Iodoacétamide 25mM final 20 mn in dark).
Incubation *overnight* with100 ng trypsin .Dessalting on ZipTip C18 column (millipore).

LC MSMS

Peptids diluted at 1:5 are purified on a capilar inverse phase column (Pepmap C18, 75 µm ID, 15 cm lenght, Dionex) , at a constant flow rate of 220 nL/mn during 90 min.

Analysis on a FTICR mass spectrometer (LTQ-FT, Thermofisher, San Jose, CA USA). Resolution 6000, between 500 and 2000 Da, followed by 7 scans MS/MS (LTQ) on the most intensives peaks. Exclusion 90 s of the fragmented precusor.

Each sample is run in triplicate.

Pontein identification

MASCOT, Bank 17D melanogaster (16535 entries).
Parameters: 2 MC, MS: 10ppm, MSMS : 1 Da, enzym = trypsin
Partial modifications: Carbamidométhylation (C), oxydation (M, H, W), Phosphorylation(Y)
Proteine identified by 1 peptid with a score >50 or 2 peptids score >30 are validated.
Only the protein identified at least in 2 out of the 3 run are considered.

Functional assay

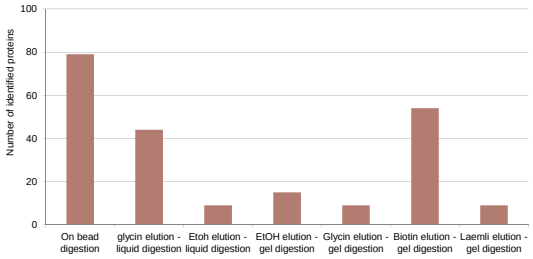
A DNA fragment of a candidate gene was obtained by PCR, using a forward and a reverse primer specific to the gene, additionally containing a T7 derived- sequence (5'-TTAATACGACTCACTATAGGGAGA-3') at the 5' end of each primer. dsRNAs were synthesized by Megascript® T7 kit according to manufacture instruction (Ambion).

After DNaseI treatment, each dsRNA was purified by RNeasy mini kit (QIAGEN). dsRNA was transfected into a S2 cell stable cell line carrying *Attacin A* luiferase reporter gene by bathing method, or was co-transfected into S2 cells with *Metchnikowin* (*Mtk*) luciferase reporter plasmid by Ca-Phosphate co-precipitation method.
After Heat killed bacteria (*E.coli* DH5a strain) stimulation, cells were harvested at 16 hours and lysed.

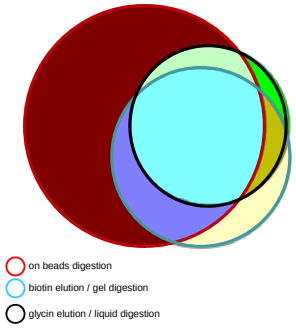
The lysate was subject to luciferase assay (Promega). The score based on *Attacin A* luciferase activity was calculated as follows:(Firefly luciferase activity arbitrary unit (FLU) of 'dsRNA transfected' with stimulation/ FLU of 'dsRNA transfected' without stimulation)/ (FLU of 'no transfection' with stimulation/ FLU of 'no transfection' without stimulation). The scores based on *Mtk*-luc activity were given as described in [4].

RESULTS

NUMBER OF IDENTIFIED PROTEINS



✓On bead digestion allows the identification of the more proteins.
✓Most of the proteins found after gel free and on-gel digestion are identified by this protocol



Overlaping of the proteins identified by the 3 most efficient protocols

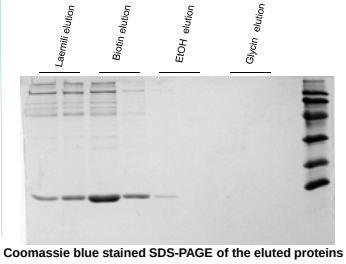
COMMENTS ON THE ELUTION PROTOCOLS

Elution with 30 mM biotine, 2% SDS, 6M urea, 2 M urea
✓Elution efficient
✓Not compatible with liquid digestion, because the SDS is difficult to eliminate
✓Compatible with SDS-PAGE purification

Elution with 30% Ethanol, 1% TFA
✓Less efficient
✓Compatible both with liquid and on gel digestion

Elution 0.1M Glycine, pH 2.5
✓Less efficient
✓Compatible both with liquid and on gel digestion
✓ Better results with liquid digestion

Elution with Laemli buffer
✓Efficient for the gel
✓Compatible only with on gel digestion



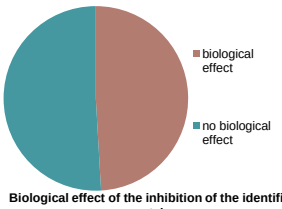
Coomassie blue stained SDS-PAGE of the eluted proteins

ON BEADS DIGESTION

✓The most efficient in terms of number of identified proteins.
✓The elution in a buffer directly compatible with LC MSMS experiment is an advantage of this protocol, saving a considerable amount of time and decreasing the loss of material.

BIOLOGICAL SIGNIFICANCE

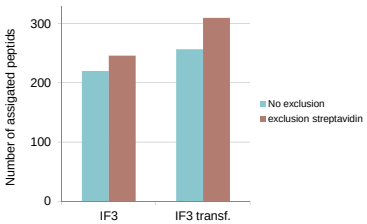
✓This assay measure the effect of the siRNA inhibition of each of 55 candidate proteins, on the immune response, by the use of a luciferase reporter [4].



Biological effect of the inhibition of the identified proteins

✓The inhibition of 27 out of the 55 (49%) proteins disturbs the immune response.
✓If we consider the proteins not identified at all in the control sample, the inhibition of 13 out of the 21(59%) have a biological effect on the immune response.

USE OF AN EXCLUSION LIST TO BYPASS THE CONTAMINATION BY THE STREPTAVIDIN PEPTIDS



Effect of the use of an exlusion list on the number of assignated peptids

✓During the trypsic digestion of proteins bound to the beads, the abundant streptavidin is also digested.

✓An elegant way to bypass the problem of the abundant streptavidin peptids for the MSMS analysis is to use an exclusion list (10 ppm for all the run).

✓In the two samples we analysed in triplicate, this exclusion list allowed to increase the number of assignated peptides

CONCLUSIONS

✓For this work, we used a complex biological model: the identification of the partners of a biotin-tagged protein.
✓Several workflows have been assessed. As described, the elution in a buffer containing biotin, SDS, urea and thiourea is very efficient [3], but is hardly compatible with gel-free digestion.
✓The on beads trypsic digestion is an interesting alternative of the elution step, wathever the buffer used. This method is faster and allow the identification of the larger number of proteins
✓The biological assay developped showed the proteins identified by the on-beads digestion are biologicaly significant.
✓Streptavidin peptides doesn't intefere with MSMS analysis, moreover they can be excluted from MSMS analysis. By this way, we could increase the proteome coverage, both by the increase of the number of identified proteins and by the increase of the number of peptids of already identified proteins.
✓Gel-free protocols offer the advantage from on-gel digestion to allow structural and functionnal analysis of prteins involved in complexes by the use of protected proteolysis [5]. This advantage is also valuable for on-beads digestion.
✓ We have also succesfully used this protocol with agarose and magnetic beads. This latter allow aa better reproducibility in the eluted volumes.
✓ In the case of immunoprecipitations, on beads digestin can also be used. However the peptides from digested antibodies are too abundant and may interfere with MSMS analysis, even with the use of an exclusion list.

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