# PROTEIN DIGESTION

## In solution digestion:

After denaturation of protein with 8 M urea, the sample was reduced and alkylated: disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 37 ºC, and then thiol groups were alkylated with 50 mM iodoacetamide for 1 h at room temperature in darkness. The sample was diluted to reduce urea concentration below 1.4 M and digested using sequencing grade trypsin (Promega, Madison, WI) overnight at 37º C using a 1:5 (w/w) trypsin/protein ratio. Digestion was stopped by the addition of 1% TFA. Whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore) or OMIX Pipette tips C18 (Agilent Technologies) or OASIS C18 columns (Waters) until the mass spectrometric analysis.

Torres, L.L., Cantero, A., Del Valle, M., Marina, A., Gallego, F.L., Guisán, J.M., Berenguer, J., Hidalgo, A. "Engineering the Sustrate Specificity of a Thermophilic Penicillin Acylase from Thermus thermophilus". *Applied and Environmental Microbiology* 2012, 79(5):1555-1562.

## In-Gel Digestion (Bands or Spots):

After drying, gel bands or spots were destained in acetonitrile:water (ACN:$H_2O$, 1:1), were reduced and alkylated (disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 56 ºC, and then thiol groups were alkylated with 50 mM iodoacetamide for 1 h at room temperature in darkness) and digested in situ with sequencing grade trypsin (Promega, Madison, WI) as described by Shevchenko et al. [1] with minor modifications [2]. The gel pieces were shrunk by removing all liquid using sufficient ACN. Acetonitrile was pipetted out and the gel pieces were dried in a speedvac. The dried gel pieces were re-swollen in 50 mM ammonium bicarbonate pH 8.8 with 12.5 ng/µl trypsin for 1 hr in an ice-bath. The digestion buffer was removed and gels were covered again with 50 mM $NH_4HCO_3$ and incubated at 37°C for 12 hr. Digestion was stopped by the addition of 1% TFA. Whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore) until the mass spectrometric analysis.

1. Shevchenko, A., Wilm, M., Vorm, O., Mann, M. "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels". *Anal Chem* 1996, 68:850-858.

2. Pérez, M., García-Limones, C., Zapico, I., Marina, A., Lienhard Schmitz, M., Muñoz, E., Calzado, Marco. A. "Mutual regulation between SIAH2 and DYRK2 controls hypoxic and genotoxic signaling pathways", *Journal of Molecular Cell Biology* 2012, 4:316-330.

## In-Gel Digestion (Stacking gel):

The protein extracts, were suspended in a volume up to 50 µl of sample buffer, and then applied onto 1.2-cm wide wells of a conventional SDS-PAGE gel (0.75 mm-thick, 4% stacking, and 10% resolving). Then run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 x 2 mm), and placed in 0.5 ml

microcentrifuge tubes [1]. The gel pieces were destained in acetonitrile:water (ACN:$H_2O$, 1:1), were reduced and alkylated (disulfide bonds from cysteinyl residues were reduced with 10 mM DTT for 1 h at 56 ºC, and then thiol groups were alkylated with 50 mM iodoacetamide for 1 h at room temperature in darkness) and digested in situ with sequencing grade trypsin (Promega, Madison, WI) as described by Shevchenko et al. [2] with minor modifications. The gel pieces were shrunk by removing all liquid using sufficient ACN. Acetonitrile was pipetted out and the gel pieces were dried in a speedvac. The dried gel pieces were re-swollen in 50 mM ammonium bicarbonate pH 8.8 with 60 ng/µl trypsin at 5:1 protein:trypsin (w/w) ratio. The tubes were kept in ice for 2 h and incubated at 37°C for 12 h. Digestion was stopped by the addition of 1% TFA. Whole supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore) or OMIX Pipette tips C18 (Agilent Technologies) or OASIS C18 columns (Waters) until the mass spectrometric analysis.

1. Moreno, M.L., Escobar, J., Izquierdo-Álvarez, A., Gil, A., Pérez, S., Pereda, J., Zapico, I., Vento, M., Sabater, L., Marina, A., Martínez-Ruiz, A., Sastre, J. "Disulfide stress: a novel type of oxidative stress in acute inflammation". *Free Radical Biology and Medicine* 2014, 70:265-277.

2. Shevchenko, A., Wilm, M., Vorm, O., Mann, M. "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels". *Anal Chem* 1996, 68:850-858.

## Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis

Peptide mass fingerprinting was conducted as previously described [1] using an Autoflex™(Bruker Daltonics, Bremen, Germany) mass spectrometer in a positive ion reflector mode employing 2,5-dihydroxybenzoic acid as matrix and an AnchorChip™surface target (Bruker Daltonics). Peak identification and monoisotopic peptide mass assignation were performed automatically using Flexanalysis™software, version 2.2 (Bruker Daltonics). Database searches were performed using MASCOT http://matrixscience.com [2] against the NCBI non-redundant protein sequence database http://www.ncbi.nih.gov. The selected search parameters were as follows: tolerance of two missed cleavages, carbamidomethylation (Cys) and oxidation (Met) as fixed and variable modifications, respectively, and setting peptide tolerance to 100 ppm after close-external calibration. A significant MASCOT probability score ($p < 0.05$) was considered as condition for successful protein identification.

1. Villar, M., Torina, A., Nuñez, Y., Zivkovic, Z., Marina, A., Alongi, A., Scimeca, S., La Barbera, G., Caracappa, S., Vázquez, J., De la Fuente, J. "Application of highly sensitive saturation labeling to the analysis of differential protein expression in infected ticks from limited samples". *Proteome Science* 2010, 8:43.

2. Perkins, DN., Pappin, DJ., Creasy, DM., Cottrell, JS. "Probability-based protein identification by searching sequence databases using mass spectrometry data". *Electrophoresis* 1999, 20:3551-3367.

# Reverse phase-liquid chromatography RP-LC-MS/MS analysis (Dynamic Exclusion Mode)

## LTQ VELOS:

The desalted protein digest was dried, resuspended in 8 µl of 0.1% formic acid and analyzed by RP-LC-MS/MS in an Agilent 1100 system coupled to a linear ion trap LTQ-Velos mass spectrometer (Thermo Scientific, Waltham, MA, USA). The peptides were separated by reverse phase chromatography using a 0.18 mm × 150 mm Bio-Basic C18 RP column (Thermo Scientific), operating at 1.8 µl/min. Peptides were eluted using a 35-min gradient from 5 to 40% solvent B (Solvent A: 0,1% formic acid in water, solvent B: 0,1% formic acid, 80% acetonitrile in water). ESI ionization was done using a microspray "metal needle kit" (Thermo Scientific) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 µscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion applied during 30 seconds periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). Database search was performed against uniprot-fungi.fasta. The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Search against decoy database (integrated decoy approach) using false discovery rate (FDR) < 0.01.

Alonso, R., Pisa, D., Marina, A., Morato, E., Rábano, A., Carrasco, L. "Fungal infection in patients with Alzheimer's disease". *Journal of Alzheimer's Disease* 2014, Vol 41:301-311, nº1.

## LTQ ORBITRAP VELOS PRO:

The desalted protein digest was dried, resuspended in 10 µl of 0.1% formic acid and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1mm × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075mm x 250 mm C18 RP column (Thermo Scientific) operating at 0.3 µl/min. Peptides were eluted using a 240-min dual gradient from 5 to 25% solvent B in 180 min followed by gradient from 25 to 40% solvent B over 240 min (Solvent A: 0,1% formic acid in water, solvent B: 0,1% formic acid, 80% acetonitrile in water). ESI ionization was done using a Nano-bore emitters Stainless Steel ID 30 µm (Proxeon) interface.
The Orbitrap resolution was set at 30.000.
Peptides were detected in survey scans from 400 to 1600 amu (1 µscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35%, and dynamic exclusion applied during 30 seconds periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). Database search was performed against uniprot-fungi.fasta. The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites,

and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Search against decoy database (integrated decoy approach) using false discovery rate (FDR) < 0.01.

Ruth Alonso, Diana Pisa, Ana Isabel Marina, Esperanza Morato, Alberto Rábano, Izaskun Rodal and Luis Carrasco. "Evidence for Fungal Infection in Cerebrospinal Fluid and Brain Tissue from Patients with Amyotrophic Lateral Sclerosis". *International Journal of Biological Sciences* 2015; 11(5): 546-558.

## Reverse phase-liquid chromatography RP-LC-MS/MS analysis (SMIM Mode)

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The mass spectrometer was operated in the selected MS/MS ion monitoring mode (SMIM mode) [1]. In this mode, the LTQ-Velos detector was programmed to perform, along the same entire gradient, a continuous sequential operation in the MS/MS mode on the doubly or triply charged ions corresponding to the peptide/s selected previously from the theoretical prediction.

The MS/MS spectra from the peptide was analyzed by assigning the fragments to the candidate sequence, after calculation the series of theoretical fragmentations, according to the nomenclature of the series as previously described [2].

1. I. Jorge, E.M. Casas, M. Villar, I. Ortega-Pérez, D. López-Ferrer, A. Martínez-Ruiz, M. Carrera, A. Marina, P. Martínez, H. Serrano, Benito-Cañas, F. Were, J. M. Gallardo, S. Lamas, J. M. Redondo, D. García-Dorado and J. Vázquez. "High-sensitivity analysis of specific peptides in complex samples by selected MS/MS ion monitoring and linear ion trap mass spectrometry: application to biological studies". *Journal of Mass Spectrometry* 2007, 42(11):1391-403.

2. Roepstorff, P. & Fohlman, J.: "Proposal for a common nomenclature for sequence ions mass spectra of pepides". *Biomed Mass Spectrom* 1984, 11, 601.

### LTQ ORBITRAP VELOS PRO:

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The Orbitrap resolution was set at 30.000.
The mass spectrometer was operated in the selected MS/MS ion monitoring mode (SMIM mode) [1]. In this mode, the LTQ-Orbitrap-Velos-Pro detector was programmed to perform, along the same entire gradient, a continuous sequential operation in the MS/MS mode on the doubly or triply charged ions corresponding to the peptide/s selected previously from the theoretical prediction.

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Nota: Los detalles específicos para cada trabajo están incluidos en los documentos MIAPEs ("Minimal Information About a Proteomic Experiment").