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Phosphoproteomic analysis of isolated mitochondria in yeast

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Summary

Mitochondria play a central role in cellular energy metabolism and cell death. Deregulation of mitochondrial functions is associated with several human pathologies (neurodegenerative diseases, neuromuscular diseases, type II diabetes, obesity, cancer). The steadily increasing number of identified mitochondrial phosphoproteins, kinases and phosphatases in last years suggests that reversible protein phosphorylation plays an important part in the control of mitochondrial processes. In addition, many mitochondrial phosphoproteins probably still remain to be identified, considering that 30% of proteins are expected to be phosphorylated in eukaryotes. In this chapter, we describe two procedures for the analysis of the mitochondrial phosphoproteome. The first one is a qualitative method that combines Blue native and Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (2D -BN /SDS-PAGE) and specific phosphoprotein staining. The second one is a quantitative approach that associates mitochondrial peptide labelling, phosphopeptide enrichment and mass spectrometry.

Keywords: *Saccharomyces cerevisiae*, mitochondria, phosphoproteome

1.Introduction

Phosphorylation is the most frequent post-translational modification: 30% of the proteins are phosphorylated at some point during their cell cycle (1). This is a reversible process which varies especially during environmental changes (2, 3), the cell cycle (4), stress, or a nutritional change (5), suggesting a role of phosphorylation in environmental adjustment. A large number of phosphorylated mitochondrial proteins have been identified and phosphorylation appears involved in various functions such as transcription, apoptosis or energy metabolism (6).

As mitochondria are a small cellular compartment, their isolation is necessary for the analysis of their phosphoproteome. This is illustrated by the work of Amoutzias et al. (7), who summarized several phosphoproteomic studies performed on yeast cells: they listed more than 10 000 sites of phosphorylation but only about one hundred were located on mitochondrial proteins. Phosphoproteomic analysis were developed using either labelling techniques with ³²P (8), detection with phosphoantibodies, fluorescent labelling with ProQ Diamond (9) or phosphorylated peptide enrichment (10, 11). In all the cases, the identification of the phosphorylated residue required analysis by mass spectrometry.

Analysis by mass spectrometry allowed a significant improvement of the knowledge of mitochondrial phosphoproteome in various organisms. However, phosphorylated proteins remain less abundant than their non-phosphorylated counterparts and the ionization of phosphopeptides is less effective than non-phosphorylated peptides, making their detection by mass spectrometry more difficult. Phosphoproteome analysis therefore requires specific analysis techniques, in particular enrichment in phosphopeptides upstream of the LC-MS / MS. The most widely used methods of enrichment are the Immobilized Metal ion Affinity Chromatography (IMAC)(10) which is based on the high affinity of phosphates to certain trivalent metal ions, and metal oxide chromatography (MOC) (11) using the titanium dioxide (TiO₂) or zirconium dioxide (ZrO₂). To optimize this enrichment step, the samples are pre-fractionated, either by SDS-PAGE, or by chromatography such as Strong Cation Exchange Chromatography (SCX) or hydrophilic interaction liquid chromatography (HILIC) (12).

These different strategies are optimal for the analysis of phosphorylated serines and threonines. Because of their presence in small quantities, phospho-tyrosines are often analysed after specific enrichment such as immunoprecipitation using an antibody against phospho-tyrosine (13). Histidine phosphorylation is not detected by standard LC-MS / MS analyzes, because they are generally conducted at acidic pH, at which phosphorylation is labile for this residue. The detection of this phosphorylated residue thus requires specific analysis strategy (14).

Finally, quantitative phosphoproteomic analysis can be performed using i) labelling methods either metabolic labelling (SILAC) (15) or chemical labelling (iTRAQ (16), multiplex stable isotope dimethyl labelling (17)) ii) Single Reaction Monitoring/ Multiple Reaction Monitoring (SRM/MRM) analysis (18).

In this chapter, we present two techniques (presented schematically in **fig.1**) that we have carried out on isolated mitochondria to analyse protein phosphorylation state. The first one relies on Blue native and Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (2D -BN /SDS-PAGE) associated to Pro-Q Diamond staining (sections 3.1 and 3.3 to 3.5). The second one is quantitative and uses multiplex stable isotope dimethyl labelling followed by fractionation by SCX and phosphopeptides enrichment by IMAC before LC-MS/MS analysis (sections 3.1, 3.2 and 3.6 to 3.9). This second approach allowed us to produce the first extensive study providing quantitative data on phosphosite responses to different carbon substrates in the yeast *Saccharomyces cerevisiae* (19).

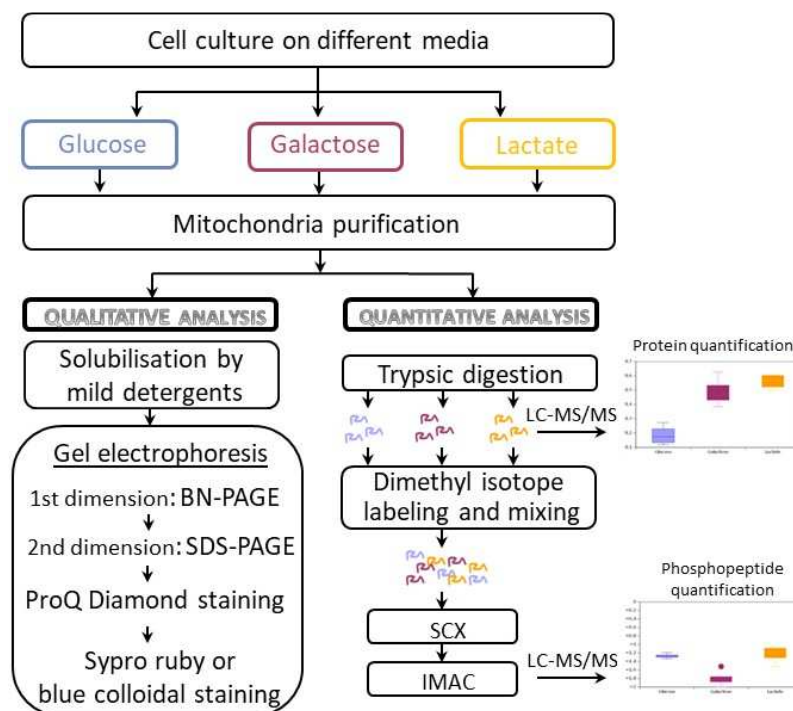


Fig.1: Scheme of two experimental approaches for the phosphoproteomic analysis of isolated mitochondria. Starting with pure yeast mitochondria, the qualitative approach permits to reveal the presence of phosphoproteins in the different complexes separated by BN-PAGE, while the quantitative approach allows the analysis of phosphopeptide abundance variations.

2. Materials

Prepare all solutions using ultrapure water (sensitivity 18 M Ω cm at 25°C).

2.1 Cell culture

Yeast WT strain was grown in galactose, glucose or lactate media (see **Note 1**).

1. Medium 1: 1.25% (w/w) bacto yeast extract, 2.5% (w/w) bacto peptone. Weigh 5g of bacto yeast extract and 10g of bacto peptone. Complete to a volume of 400mL with water. Autoclave at 120°C, 20 min.
2. 10% galactose: weigh 10g of galactose. Complete to a volume of 100mL with water. Autoclave at 120°C, 20 min.
3. 10% glucose: weigh 10g of glucose. Complete to a volume of 100mL with water. Autoclave at 120°C, 20 min.
4. Galactose medium: 1% (w/w) bacto yeast extract, 2% (w/w) bacto peptone, 2% (w/w) galactose. Just before use, mix 400mL of medium 1 and 100mL of 10% galactose.
5. Glucose medium: 1% (w/w) bacto yeast extract, 2% (w/w) bacto peptone, 2% (w/w) glucose. Just before use, mix 400mL of medium 1 and 100mL of 10% glucose.
6. Lactate medium: 0.5% (v/v) lactic acid, 0.75% (w/w) bacto yeast extract, 0.75% (w/w) bacto peptone. Sample 5.9 mL of lactic acid. Add 800 mL of water. Adjust the pH to 4 with a solution of 10N KOH. Then add 7.5g of bacto peptone and 7.5g of bacto yeast extract. Check the pH to be 4.5. Add water to a volume of 1L. Autoclave at 120°C, 20 min.

2.2 Preparation of mitochondria by differential centrifugation

1. DTT buffer: 100 mM TRIS-H₂SO₄ pH 9.4 and 10 mM DTT (see **Note 2**).
2. 0.1M Potassium phosphate buffer pH 7.4: mix 80.2 mL of 1M K₂HPO₄ and 19.8 mL of 1M KH₂PO₄. Complete to a volume of 1L with water.
3. Zymolyase buffer: 1. 2M sorbitol, 20mM potassium phosphate, pH7.4.
4. Zymolyase-20T.
5. PMSF 100 mM: weigh 17.4mg of PMSF in 1 mL of ethanol. (see **Note 3**)
6. Homogenization buffer: 0.6M sorbitol, 10 mM TRIS-HCl pH7.4, 1 mM EDTA, PMSF 1mM.

2.3 Preparation of mitochondria purified on sucrose gradient

1. SEM buffer: 250mM sucrose, 1mM EDTA, 10mM MOPS-KOH pH 7.2
Weigh 2.57g of sucrose. Add 3 mL of 100 mM MOPS-KOH pH 7.2 and 100 μ L of 0.3M EDTA pH 7.4. Complete to a volume of 30mL with water.
2. EM buffer: 1mM EDTA, 10mM MOPS-KOH pH 7.2
Add 100 mL of 100 mM MOPS-KOH pH 7.2 and 3.33 mL of 0.3M EDTA pH 7.4. Add water to a volume of 1L.
3. Solutions for sucrose gradient:
 - 3.1 60% sucrose solution: weigh 12g of sucrose and add EM buffer to a volume of 20 mL.
 - 3.2 32% sucrose solution: weigh 16g of sucrose and add EM buffer to a volume of 50 mL.
 - 3.3. 23% sucrose solution: weigh 4.6g of sucrose and add EM buffer to a volume of 20mL.
 - 3.4 15% sucrose solution: weigh 3g of sucrose and add EM buffer to a volume of 20 mL.

2.4 Solubilisation of mitochondria

1. Gel Buffer (3x): 1.5 M ϵ -amino-n-caproic acid 0.5M, 150 mM Bis-Tris. The pH is adjusted to 7 with HCl at 4°C. The buffered is filtered on 0.45 μ m filter.
2. DNase solution: 0.2 mg/mL DNase , 5 mM MgCl₂ and 1 mM PMSF were added in gel buffer (x3).
3. Solubilization buffer: 2% laurylmaltoside (weight/volume) or 2% digitonine (weight/volume) were prepared in gel buffer (x3).

2.5 BN/ SDS PAGE – 2D electrophoresis

2.5.1 2D-electrophoresis – First dimension: BN-PAGE

1. Acrylamide/Bis solution: 40% solution 29:1
2. Ammonium persulfate: prepare a 10% (w/v) solution in water and store at 4°C for up to one month.
3. 5% acrylamide solution: Mix 625 μ L of acrylamide:bis-acrylamide solution and 1.67 mL of gel buffer (x3). Complete to 5 mL with H₂O. Add 3 μ L of Temed and 30 μ L of 10% ammonium persulfate.

4. 10% acrylamide solution: Mix 1.3 mL of acrylamide:bis-acrylamide solution and 1.67 mL of gel buffer (x3). Complete to 5 mL with H₂O. Add 2.5 μL of Temed and 25 μL of 10% ammonium persulfate.
5. Stacking gel (3%): Mix 375 μL of acrylamide: bis solution and 1.67 mL of gel buffer (x3). Complete to 5 mL with H₂O. Add 5 μL of Temed and 50 μL of 10% ammonium persulfate.
6. Cathode buffer A: 15 mM Bis-Tris, 50 mM Tricine. The pH is adjusted to 7.0 with a concentrated HCl solution at 4°C. The buffer is filtered on Millipore 0.45 μm filter. Then 0.02% (w/v) Serva Blue G-250 is added and the buffer is sonicated in a bath sonicator to solubilize the Serva Blue G-250.
7. Cathode buffer B: same as cathode buffer A except that no Serva Blue G-250 is added.
8. Anode buffer: 50 mM Bis-Tris. The pH is adjusted to 7.0 with a concentrated HCl solution at 4°C. The buffer is filtered on 0.45 μm filter.
9. Molecular weight markers: High Molecular Weight Calibration Kit for Native Electrophoresis.

2.5.2 2D-electrophoresis – Second dimension: SDS-PAGE

1. Acrylamide/Bis solution: 40% solution 37.5:1.
2. Resolving gel buffer: 3M Tris-HCl pH8.8. Weigh 363 g Tris. Add water to a volume of 700 mL. Mix and adjust pH to 8.8 with HCl. Complete to 1L with water. Filter through a 0.45 μm filter. Store at 4°C.
3. Stacking gel buffer: 0.5M Tris-HCl, pH 6.8. Weigh 60.5g Tris and prepare a 1L solution as in previous step. Filter through a 0.45 μm filter. Store at 4°C.
4. 10% acrylamide solution: Measure 2.5 mL of acrylamide: bis-acrylamide solution and 1.25 mL of Tris-HCl 3M pH8.8. Add 3 μL of Temed and 30 μL of 10% ammonium persulfate. Complete with H₂O to a volume of 10 mL.
5. 20% acrylamide solution: Measure 5 mL of acrylamide: bis-acrylamide solution and 1.25 mL of Tris-HCl 3M pH8.8. Add 2.5 μL of Temed and 22.5 μL of 10% ammonium persulfate. Complete with H₂O to a volume of 10 mL.
6. Stacking gel (5%): Measure 1.5 mL of acrylamide: bis-acrylamide solution and 3 mL Tris-HCl 0.5M pH 6.8. Add 20 μL of Temed and 200 μL of 10% ammonium persulfate. Complete with H₂O to a volume of 12 mL.
7. Tris- glycine (x5) buffer: Tris 250 mM and glycine 1.92M pH 8.8. Weigh 60.5g Tris and 288g of glycine. Complete with H₂O to a volume of 2L. pH will be around 8.8 without any adjustment.
8. Cathode Buffer: 50 mM Tris, 384 mM Glycine, 0.1% SDS, 1 mM EDTA: dilute x5 Tris-glycine (x5) buffer and add 0.1% SDS (w/v) and 1 mM EDTA.
9. Anode Buffer: 50 mM Tris, 384 mM Glycine; dilute x5 Tris-glycine (x5) buffer
10. Molecular weight markers: Precision Plus Protein Standards, dual color.

2.6 Phosphoproteins staining

1. ProQ Diamond stain.
2. Fixation solution: 50% methanol, 10% acetic acid, 40% water (see **Note 4**).
3. Discoloration solution: 50 mM ammonium acetate, 20% acetonitrile, 80% water.
4. Molecular Weight Standards: Peppermintstick Phosphoprotein.

2.7 Proteins staining

After phosphoproteins staining, proteins could be stained either with Sypro Ruby protein gel stain or Coomassie Blue protein gel stain.

1. Syproruby stain.
2. Discoloration solution: 7M acetic acid, 10% methanol, 83% water.
3. Coomassie Blue stain.

2.8 Preparation and labelling of mitochondrial peptides for quantitative mass spectrometry experiment

2.8.1. Digest sample

1. Zwitterionic acid labile surfactant ZALS I.
2. Ammonium bicarbonate (NH_4HCO_3).
3. ZUT buffer : 0.1% ZALS I solubilised in 50 mM NH_4HCO_3 , 6M urea, 2M thiourea, 30 mM Tris-HCl pH 8.8, 10mM DTT.
4. 2D Quant kit.
5. Sequencing grade modified trypsin.
6. Trypsin digestion: trypsin/protein ratio: 1/30.
7. Trifluoroacetic acid.
8. Vacuum centrifuge (Speed vac concentrator)

2.8.2. Stable isotope dimethyl labeling of tryptic peptides

! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic; prepare solutions in a fume hood.

1. Formaldehyde solution for molecular biology, (36.5-38% in H_2O).
2. Formaldehyde (CD_2O) (20%, 98% D).
3. Formaldehyde ($^{13}\text{CD}_2\text{O}$) (20%, 99% ^{13}C , 98% D).
4. Sodium cyanoborohydride (NaBH_3CN).
5. Sodium cyanoborodeuteride (NaBD_3CN).
6. Sodium phosphate buffer pH 7.5: mix 1mL of 50mM NaH_2PO_4 with 3.5 mL of 50 mM Na_2HPO_4 ; Sodium phosphate monobasic (NaH_2PO_4). Sodium phosphate dibasic (Na_2HPO_4).
7. Light labelling solution: Add 5% (v/v) formaldehyde 4%, 5% (v/v) cyanoborohydride 0.6M in 50mM sodium phosphate buffer pH 7.5.

8. Intermediate labelling solution: Add 5% (v/v) formaldehyde ²D 4%, 5% (v/v) cyanoborohydride 0.6M in phosphate buffer pH 7.5.
9. Heavy labelling solution: Add 5% (v/v) formaldehyde ²D, ¹³C 4%, 5% (v/v) cyanoborodeutéride 0.6M in phosphate buffer pH 7.5.
10. Buffer A: 0.6% acetic acid in ultrapure water.
11. Buffer B: 0.6% acetic acid in 80% (v/v) acetonitrile.
12. Sep-Pak C18 3 cc Vac RC Cartridge 500mg.
13. Vacuum manifold system.
14. Vacuum centrifuge (Speed vac concentrator).
! CRITICAL Labelling reagent mixtures should be kept at 4°C and not stored longer than 24 h to ensure labelling efficiency.

2.9 Two-steps phosphopeptides enrichment

2.9.1 Strong cation exchange chromatography fractionation

1. Buffer C: 30% (v/v) acetonitrile, 0.5% (v/v) formic acid in ultrapure water (pH 2.5)
2. Buffer D: 30% (v/v) acetonitrile, 0.5% (v/v) formic acid and 540 mM ammonium formate in ultrapure water (pH 4.7)
3. HPLC: Ultimate LC system combined with a Famos autosampler and a Switchos II microcolumn switch system.
4. Collector: Probot system.
5. SCX column: Zorbax 300 SCX, 2.1mm ID x 150 mm.

2.9.2 Enrichment of phosphopeptides by Immobilized Metal ion Affinity Chromatography (IMAC)

1. Buffer E (equilibration buffer): 30% (v/v) acetonitrile, 250 mM acetic acid in water.
2. Buffer F (elution buffer): 30% (v/v) acetonitrile, 0.4M ammonium hydroxide in water.
3. PHOS-Select iron affinity gel.
4. SigmaPrep Spin-Columns.
5. Centrifuge.
6. Vacuum centrifuge (Speed vac concentrator).

2.10 LC-MS/MS analysis

1. Buffer G: 2% acetonitrile, 0.05% trifluoroacetic acid and 0.05% formic acid.
2. Buffer H: 2% acetonitrile and 0.1% formic acid.
3. Buffer I: 0.1% AF H₂O 100%.
4. Buffer J : 0.1% AF CH₃CN 100%.
5. NanoHPLC : NanoLC-Ultra system.
6. Mass spectrometer: Q-Exactive mass spectrometer with a nano-electrospray interface.
7. Pre-column cartridge: PepMap 100 C18 (particles of 5µm, 100 µm i.d., 10 mm length).
8. Analytical column: PepMap 100 C18 (particles of 3 µm; 75 µm i.d., 150 mm length).

3. Methods

3.1 Preparation of mitochondria by differential centrifugation

Yeast wild type strain is grown either in 2% glucose medium or 2% galactose medium to an $OD_{600nm} = 6$, or 2% lactate medium to an $OD_{600nm} = 4.5$.

The following protocol is the one developed by Meisinger et al. (20) with slight modifications.

1. Harvest the cells by centrifugation at 3000g for 10 min at 4°C and wash with distilled water
2. Resuspend the cells in 2mL/ g (wet weight) DTT buffer (see **Note 5**) and shake at 30°C, 20 min in an incubator (Infors -120rpm)
3. Centrifuge at 3000g , 5 min
4. Wash the pellets in zymolyase buffer
5. Centrifuge at 3000g , 5 min
6. Resuspend the pellets in 7mL/g (wet weight) of zymolyase buffer
7. Digest the cell wall enzymatically with Zymolyase-20T at 3mg/g of cells at 30°C for about 1H. (see **Note 6**)
8. Centrifuge at 3000g , 5 min at 4°C
9. Resuspend the pellets in 6.5mL/g (wet weight) of homogenisation buffer (see **Note 7**)
10. Homogenize with 5 pestle strokes in a glass-teflon potter
11. Centrifuge at 1500g , 5 min at 4°C to eliminate cell debris and nuclei
12. Keep the supernatant and centrifuge at 4000g , 5 min at 4°C
13. Keep the supernatant and centrifuge at 12000g, 15 min at 4°C in order to harvest the crude mitochondria.

3.2 Preparation of mitochondria purified on sucrose gradient

Steps 1 to 4 follow the protocol developed by Meisinger et al. (20).

1. Resuspend the crude mitochondria pellet in 0.5 mL of SEM buffer containing 1mM PMSF, to have a final concentration of mitochondria of 5 mg/mL approximately.
2. Homogenize the mitochondria with 10 up-and-down strokes in a Potter homogenizer and load on a three-step sucrose gradient (1.5mL 60%, 4mL 32%, 1.5mL 23% and 1.5mL 15%).
3. Centrifuge the sucrose gradient at 134 000 g at 4°C during 1h in a SW41 Ti swinging bucket.
4. Collect the purified mitochondria at 60%-32% interface and resuspend in 1mL of SEM buffer containing 1mM PMSF to have a final concentration of mitochondria of 1 mg/mL approximately.
5. Measure the protein concentration of purified mitochondria using the 2-D Quant Kit with BSA as a standard.
6. Aliquot the solution of purified mitochondria in 2mL safe lock tubes.

7. Centrifuge the aliquots at 14000g at 4°C during 15 min; throw the supernatants.
8. Then, in order to avoid degradation of proteins and to limit dephosphorylation events, precipitate the proteins by adding 2 mL of TCA 10% in each tube.
9. Homogenize the protein solutions by inversion of the tubes and incubate at 4°C during 15 min.
10. Centrifuge the protein solutions at 14000g at 4°C during 15 min; throw the supernatants.
11. Add 2 mL of acetone (-20°C) in every tube to eliminate the excess of TCA and transfer the protein solutions in Corex tubes.
10. Homogenize the protein solutions and centrifuge at 14000 g at 4°C during 15 min; Throw the supernatants.
11. Dry the protein precipitates and store at -20°C.

3.3 BN-PAGE/ SDS - 2D electrophoresis

Method for non-denaturing gels was initially developed in (21)

3.3.1 Solubilization of mitochondria

1. Prior to their solubilisation, treat the mitochondria with DNase: resuspend in order to have a final concentration of mitochondria equal to 1 mg/mL in DNase solution. Incubate the sample 30 min at 25°C.
 2. Centrifuge the sample at 13 000g at 4°C during 15 min.
 3. Resuspend the pellet of mitochondria in solubilisation buffer and incubate at 4°C during 30 min.
 4. Centrifuge the sample at 100 000g and at 4°C during 30 min. Throw the pellet.
 5. Add Serva Blue G in the supernatant in order to have a final concentration of 0.25%.
- (See **Note 8**).

3.3.2 Preparation of BN-PAGE

1. Prepare a 5%-10% acrylamide gradient resolving gel in a 8 cm x 6.5 cm x 0.10 cm gel cassette.
2. Load a 3% acrylamide stacking gel on the resolving gel. Then immediately insert a 10-well gel comb.
3. Load the samples in the wells: the volume of samples is calculated to have 25µg of proteins/lane. After the loading of samples, completely fill the wells with cathode buffer A.
4. Perform electrophoresis at 4°C.
5. During the first electrophoresis period, cathode buffer is buffer A. Perform electrophoresis at 100V constant during 1h then at 200V constant during 1h more.

6. During the second electrophoresis period, cathode buffer is buffer B. Perform electrophoresis at 200V until the dye front has reached the bottom of the gel.

3.3.3 Preparation of the sample for the second dimension

Immediately following BN-PAGE, cut a lane of interest and incubate it in 60mM Na_2CO_3 , 60 mM DTT and 2% SDS during 30 minutes.

3.3.4 Preparation of SDS-PAGE

1. Prepare a 10%-20% acrylamide gradient resolving gel in a 8 cm x 5 cm x 0.15 cm gel cassette.
2. Load a 5% acrylamide stacking gel on the polymerised resolving gel. Insert a preparative comb containing a wide single well and a narrow reference.
3. Carefully load one BN-PAGE lane, previously incubated as described in 2.3.3., on the gel to avoid any bubble and pour the resolving gel on the lane. Load the protein standards in the reference well.
4. Perform electrophoresis at 4°C and 120V constant until the dye front has reached the bottom of the gel.

3.4 ProQ Diamond staining (see Fig.2)

1. Following electrophoresis, incubate the gel in the fixation solution, during 1h at least.
2. Then, wash 3 times with water; each wash lasting 15 minutes.
3. Incubate with the Pro-Q Diamond during 2h in the darkness.
4. Incubate three times in the discoloration solution in the darkness; each incubation lasting 30 minutes.
5. Wash 2 times with water in the darkness; each wash lasting 5 minutes.
6. Phosphoproteins are then detected by fluorescence detection: the excitation maximum of Pro-Q Diamond is at 550 nm and its emission maximum at 580 nm.

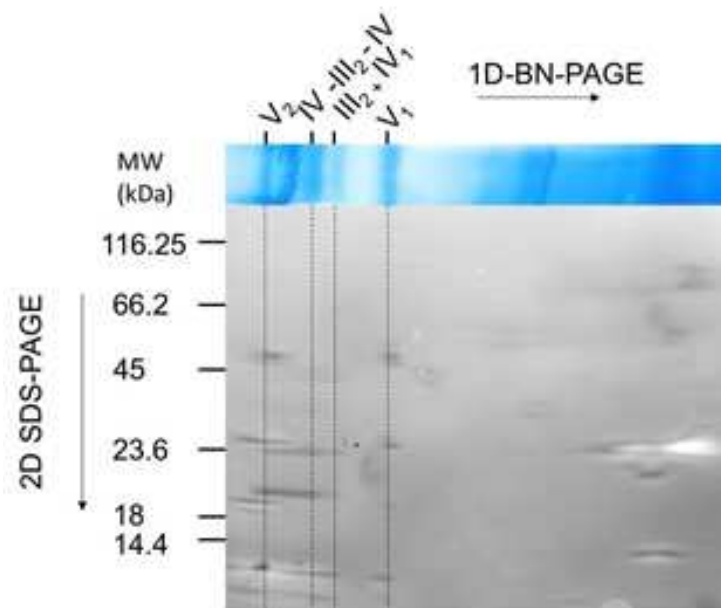


Fig.2: 2D BN /SDS-PAGE electrophoresis. Mitochondria were separated in the first dimension on BN-PAGE after digitonin solubilisation. Mitochondrial complexes (monomeric [V₁] and dimeric [V₂] complex V) and supercomplexes (IV-III₂-IV and III₂-IV₁) subunits are then separated in a second dimension on SDS-PAGE and phosphoproteins were detected after ProQ Diamond staining.

3.5 Sypro Ruby staining

1. Following Pro-Q Diamond detection, incubate the gel in Sypro Ruby during 1 night in the darkness.
2. Incubate three times in the discoloration solution in the darkness; each incubation lasting 30 minutes.
3. Wash 2 times with ultrapure in the darkness, each washing step lasting 5 minutes.
4. Proteins are then detected by fluorescence detection: the two excitation maxima of Sypro Ruby are at 280 and 450 nm and its emission maximum at 610 nm.

3.6 Preparation and labeling of mitochondrial peptides for quantitative mass spectrometry experiment

3.6.1. Suspension

1. Dry the protein precipitates in corex tubes under vacuum during 15 min.
2. Resuspend the proteins in 400 μ L of ZUT buffer in global, mix by vortex and apply short spin centrifuge (i.e. 200 rpm). Additional 200 μ L of ZUT buffer can be added if necessary (see **Note 9**).
3. Collect the protein solutions and measure the protein concentration with the 2D Quant kit

3.6.2. Alkylation

1. Add 1M iodoacetamide in each sample to have a final concentration of iodoacetamide equal to 40 mM.
2. Incubate the samples 1h in darkness.

3.6.3. In-solution trypsin digestion

1. Dilute the samples with 7 volumes of 50mM of ammonium bicarbonate.
2. Add trypsin in samples in order to have a trypsin/protein ratio of 1/30. Before its addition in samples, resuspend the trypsin with 50mM ammonium bicarbonate (200µL ammonium bicarbonate for 40µg of trypsin).
3. Incubate the samples overnight at 37°C.
4. Stop the digestion by addition of acid in order to reach pH 2.
5. Dry the samples under vacuum using a speed vac.

3.6.4. Stable isotope on column labelling

The multiplex stable isotope dimethyl labelling procedure is those developed by Boeserma et al. (17)

! CAUTION Formaldehyde solutions and formaldehyde vapors are toxic; place the vacuum manifold in a fume hood. The vacuum manifold solvent liners should be inert and disposable to prevent carry-over between experiments.

1. Samples are suspended with formic acid 5% (approximately pH 2).
2. Labelling of samples is made on Sep-Pak C18 columns (see 2.7.2). The pressure must not exceed 5 mmHg (vacuum manifold system). Columns are prepared with 2mL of acetonitrile and 2x2mL of buffer A.
3. Samples are loaded on columns
4. Columns are washed with 2 mL of buffer A.
5. Peptides fixed in the columns are labelled with 7 x 1 mL of the appropriate labelling solution. For complete labeling, make sure that step takes at least 10 min.
6. Columns are washed with 2mL of buffer A.
7. Labelled peptides are eluted with 2 x 500µL of buffer B.
8. Labelled peptides issued from different samples are mixed at a 1:1 ratio and dried by vacuum centrifugation.

3.7 Two-steps phosphopeptide enrichment

3.7.1 Strong exchange chromatography fractionation

1. Prior to the phosphopeptides enrichment, separate the peptides with an SCX chromatography coupled to an UV detection system (flux: 200 μ L/min; run last 80 min).
2. Resuspend the labeled samples in 500 μ l of buffer C.
3. Equilibrate the cation-exchange column by washing with buffer C (see **Notes 10 and 11**).
4. Load the sample on the column.
5. Wash with buffer C, over 14 min.
6. Elute the sample using a linear gradient from 0% to 65% of buffer D during 40 min.
7. During the run, the occurrence of peptide at the outlet of the column is continuously analyzed with an on-line UV detection cell.
8. Systematically collect the eluted fraction in a 96-well sample collection plate (300 μ l/fraction).
9. Thanks to UV profile, select the wells where UV profile indicates the presence of peptides.
10. Mix the wells for a total of 12 fractions. Pooling too many wells would increase peptide concentrations in each extract and decrease the efficiency of the IMAC enrichment step. For the further steps, a fraction means a pool of 2 or 3 wells.
11. Lyophilize each fraction in a speed vac evaporator.

3.7.2 Enrichment of phosphopeptides by Immobilized Metal ion Affinity Chromatography

1. Before the phosphopeptides enrichment, PHOS-Select Iron affinity gel is prepared as following:
 - 1.1. Homogenize the resin in glycerol
 - 1.2. Centrifuge at 500 g and at room temperature during 2 min: supernatant is thrown
 - 1.3. Resuspend the resin in the Equilibration buffer and washed 4 times with this buffer
2. Resuspend each fraction with 300 μ L of equilibration buffer and add 80 μ L of resin. Incubate 1hour with mixing at room temperature (end over-end rotation recommended).
3. Load each fraction on one spin column and make a short spin centrifugation until 5000g.
4. Wash the spin column two times with 200 μ L of equilibration buffer and one time with 200 μ L of ultrapure water (short spin centrifugation until 5000g).
5. Elute the phosphopeptides with 2 x 30 μ L of elution buffer: make a first short spin centrifugation until 2000g and a second one until 14000g (see **Note 12**).

3.8 Phosphopeptide analysis LC-MS/MS

1. Resuspend the fractions in 20 μL of buffer G.
2. Load 4 μL at 7.5 $\mu\text{L}/\text{min}$ on the pre-column cartridge with buffer H.
3. Desalt the peptides with solvent I during 3 min.
4. Load the peptides on the analytical column and separate by a linear gradient from 5% to 30% buffer J during 37 min at 300nl/min.
5. Analyse the eluted peptides with mass spectrometer using Xcalibur 2.1 interface.
6. Data-dependent acquisition of peptide ions included a full MS scan covering the 300 to 1400 mass-to charge ratios (m/z) with a resolution of 70000 and a MS/MS step (normalized collision energy: 30%; resolution: 17500). The MS/MS step was reiterated for the 8 major ions detected during the full MS scan. The dynamic exclusion was set to 45 s.

3.9 Bioinformatics Data Analysis

1. Conversion of raw files to mzML format (ProteoWizard software V3.0.3706).
2. Database searches process: use the XtandemPipeline (<http://pappso.inra.fr/bioinfo/xtandempipeline/>) to run database search with the X!Tandem search engine (22) and to manage redundancy (grouping of phosphopeptides and proteins harboring the same phosphosite(s)). Export data in MassChroQ format
3. Quantification: use the MassChroQ software (23) to quantify phosphopeptides according to their extracted ion current (XIC). Align LC-MS runs performed on the same SCX fraction number.
4. Computation of relative quantitative changes: for each phosphopeptide, compute the ratios between light, intermediary and heavy isotopes (if one of the isotopes is chosen as a reference, 2 ratios are computed, the reference being the common denominator). Normalize the ratios by dividing ratios by the median value of all phosphopeptide ratios within each triplex or duplex. Use log-transformed ratios for subsequent statistic tests.
5. When protein abundances were analysed in the same samples, phosphopeptide intensities can also be normalized by protein amounts, to take into account their possible variation in the computation of phosphopeptide quantitative changes.

3.10 Conclusion

The methods presented in this chapter are complementary means of studying the mitochondrial phosphoproteome. The qualitative method allows the analysis of the relation between the state of phosphorylation of proteins and their interaction with other proteins within a complex or within complexes interacting together (supercomplexes, for example). The quantitative method allows a comprehensive study on the state of phosphorylation of mitochondrial proteins independently from their possible interactions with each other, but allows the analysis of the effects of different conditions (carbon substrate, aerobic / anaerobic) or mutants.

4. Notes

1. Cells were grown in liquid medium with shaking (130 rpm) at 28°C in a standard incubator.
2. DTT buffer has to be prepared ex-temporarily.
3. PMSF is added in buffer just before its use; its stability in aqueous buffer solutions doesn't exceed 1h.
4. Be careful: methanol can't be substituted by ethanol.
5. Pre-equilibrate the DTT buffer at 30°C before use.
6. The enzymatically cell wall digestion is followed by measurement of OD at 600nm, until it reaches 10% of the initial value.
7. Precool the homogenisation buffer at 4°C.
8. Solubilized mitochondria can be stored at -20°C before loaded on BN-PAGE gel.
9. To simultaneously analyse different samples, concentrations of each sample are compared in order to have the same quantity of proteins in the different samples. If samples' volumes are not the same, they will be adjusted with ZUT buffer.
10. Start degassing buffer C and buffer D 30 min before the start of SCX chromatography.
11. Purge channels A and B, respectively connected to buffer C and buffer D, of the SCX chromatograph before its use.
12. The elution step in the IMAC enrichment protocol must be done quickly: in the presence of elution buffer, resin is not stable more than 5 minutes.

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