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Mitochondria proteome profiling: A comparative analysis between gel- and gel-free approaches

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ABSTRACT

Mitochondrial proteomics emerged aiming to disclose the dynamics of mitochondria under various pathophysiological conditions. In the present study we investigated the relative merits of gel-based (2DE and SDS-LC) and gel-free (2D-LC) protein separation approaches and protein identification algorithms (Mascot and Paragon) in the proteome profiling of mitochondria isolated from cultured fibroblasts, a sample traditionally used for diagnosis purposes.

Combining data retrieved from 2DE, 2D-LC and SDS-LC and search methods, a total of 696 non-redundant proteins were identified. An overlap of only 19% between the proteins identified by the three different methods was observed when Mascot and Paragon were used. Regarding protein ID, a consistency in the number of identified proteins *per* sample was noticed for 2DE approach. Independent of the methodological approach chosen, it was noticed that the predominance in mitochondria of hydrophilic proteins with 20–50 kDa and p*I* 5–6 and 8–9; however, 2D-LC and SDS-LC allowed the enrichment of proteins with a mass below 30 kDa and of basic proteins with p*I* values above 8. In conclusion, data from the present study highlight the power of integrating different separation technologies and protein identification algorithms.

1. Introduction

Mitochondrial dysfunctions are associated with many pathophysiological conditions, such as metabolic disorders, diabetes mellitus, cancer, neurodegenerative diseases and aging [1,2]. Among these, metabolic disorders involving defects in proteins/enzymes from pathways harbored in mitochondria are rare but related with high morbidity and mortality [3]. Many effects of these disease processes have been studied using classic biochemical methods focused on a particular protein [4]. Recent developments in proteomics have allowed a global perception into protein expression, localization, and interaction, and how they are modulated by pathophysiological conditions. Indeed, proteome profiling has been intensely used for the investigation of pathogenic mechanisms and functional correlations on protein levels in a non-biased manner [2,4,5]. Among the protein separation procedures used for mitochondria proteome profiling, two-dimensional gel electrophoresis (2DE) as well as multidimensional liquid chromatography (2D-LC) have been adopted [1,6–8]. While 2DE, a classical proteomic technology, separates proteins based on their isoelectric point (p*I*) and molecular weight (MW) with a limited dynamic range, 2D-LC partially overcomes the shortcomings of 2DE such as limitations in detecting proteins with extreme alkalinity, hydrophobicity, and molecular mass, allowing large-scale, discovery-driven proteomics [1,4,9–11].

Cultured skin fibroblasts are an attractive sample for diagnostic testing and research of metabolic diseases involving defects in mitochondrial proteins, considering the minimally invasive character of sampling and the large amount of cell material that can be obtained by culturing [12]. For the diagnosis of certain diseases, enzymatic measurements or molecular genetic analysis of tissue and/or cell samples is required [3] and for that, tissue biopsy is often needed. Although tissues like heart, skeletal muscle and liver are among the main targets of metabolic disorders, such as fatty acid oxidation defects [13], skin biopsy is usually preferred. Besides being ethically straightforward, the isolated fibroblasts can be used to measure ATP synthesis, oxygen consumption, electric membrane potential, and substrate oxidation rates [3,12,13]. Moreover, fibroblasts can be stored and retested when desired and might be used as a model system to study diseases' pathogenesis [3]. Despite all these advantages, few studies have

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been performed focused on mitochondrial protein profiling in cultured fibroblasts [1,6,7] and their use might be advantageous in the investigation of complex defects where several factors may contribute to disease [1].

In order to clarify the strengths and limitations of proteomics to study the mitochondrial proteome in fibroblasts and the potential implications for the comprehension of metabolic diseases' pathogenesis, we performed a comparative analysis of gel-based and gel-free protein separation approaches, and protein identification algorithms (Mascot and Paragon). We report the advantages of combining multidimensional approaches to get a deep protein profile screening of mitochondria isolated from cultured fibroblasts aiming a more comprehensive and effective manner to investigate the mitochondrial proteome. Nevertheless, from the separation approaches tested, a higher number of identified mitochondrial proteins were achieved with SDS-LC approach, apart from the protein identification algorithm chosen.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all reagents such as triethylammonium bicarbonate (TEAB), trifluoroacetic acid (TFA), protease inhibitor cocktail, formic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), urea, CHAPS were purchased from Sigma-Aldrich. Immobiline drystrip and ampholytes were from GE Healthcare. Sequencing grade modified trypsin (bovine) was from ABSciex (ABSciex, USA).

2.2. Cell culture

Skin fibroblasts from healthy individuals ($n=3$) were grown in Ham F10 nutrient mixture supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 1% penicillin, streptomycin and fungizone, in 75 cm^2 culture flasks. Ten culture flasks from each sample were grown to subconfluence before mitochondria isolation.

2.3. Mitochondria isolation and protein extraction

Isolation of mitochondria from the cell pellet was performed at 4 °C according to Schwab et al. [14] with minor modifications. Briefly, the cell pellet was suspended in isolation buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES and 5 g/L BSA, pH 7.5) and was then centrifuged at 500 × g for 2 min. The supernatant was discarded and the remaining pellet was suspended in 500 μL of isolation buffer. The cell suspension was homogenized in a tight-fitting Potter homogenizer (Teflon pestle). After centrifugation at 1500 × g for 10 min, the supernatant was kept on ice. The pellet was homogenized and centrifuged as described above. The two supernatants were pooled and centrifuged at 10,000 × g for 10 min. The resulting mitochondrial pellet was washed with 100 μL of BSA-free isolation buffer. Mitochondrial proteins were extracted according to the different separation procedures tested and protein content was determined with RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Following fibroblast mitochondria isolation, three different procedures were used for protein characterization (Fig. 1).

2.4. Two-dimensional electrophoresis (2DE)

Two-dimensional electrophoresis (2DE) was performed as previously reported with minor modifications [6]. In brief, 250 μg of mitochondrial protein extract were diluted to 250 μL with a rehydration solution containing 8 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer (pH 3–10 NL), 0.2% DTT and loaded on 13 cm IPG strips (pH 3–10 NL; GE Healthcare). Isoelectric separation was performed using the following focusing program: 12 h at 50 mV (rehydration), 2 h at 150 V (gradient), 1 h at 500 V (gradient), 1 h at 1000 V (gradient) and 3 h at 8000 V ("step-n-hold"). After IEF separation, the gel strips were applied on top of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (12.5%) and proteins were separated at 200 V constant current until the bromophenol blue front reached the bottom of the gel. At least three gels were generated for further gel image analysis and mass spectrometry characterization. The gels were stained with Coomassie Colloidal-G250, and scanned with Gel Doc XR (Bio-Rad). The "Spot Detection Wizard" function in PdQuest software (v8.0.1, Bio-Rad) was used to automate the process of finding protein spots and based on the automated spot detection results, misdetected spots were added or removed using visual inspection. All detected spots were manually excised for in-gel digestion.

2.5. One-dimensional gel electrophoresis (SDS-PAGE)

Seventy micrograms of protein were incubated with SDS sample buffer (10% (w/v) sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 6.8), 4% glycerol, bromophenol blue (w/v)) for 5 min at 100 °C. Samples were loaded onto Laemli gel (12.5%) and electrophoresis was carried out at a constant voltage of 200 V current until the bromophenol blue front reached the bottom of the gel. Gels were stained with Coomassie Colloidal-G250. Following image acquisition, the entire gel lanes were manually cut out lengthwise and divided into 16 gel slices for in-gel digestion.

2.6. In-gel/band protein digestion

Annotated spots or bands were in-gel digested with trypsin. Briefly, gel pieces were washed three times with 25 mM ammonium bicarbonate/50% acetonitrile and further dried in a Speed-Vac. Then, 10 μL of 25 μg/mL modified bovine trypsin (ABSciex, USA) in 25 mM ammonium bicarbon ate was added to the dried gel pieces and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by the addition of 10% formic acid/50% acetonitrile, which was repeated three times, followed by dryness in a Speed-Vac. 2DE spot's tryptic peptides were resuspended in acetonitrile/formic acid solution and mixed (1:1) with a matrix consisting of α-cyano-4-hydroxycinnamic acid (5 mg in 1 ml of 50% acetonitrile/0.1% trifluoracetic acid). Aliquots of samples were spotted onto MALDI sample target plates. SDS-PAGE bands tryptic peptides were resuspended in acetonitrile/trifluoroacetic acid (5% and 0.01%, respectively) for reverse-phase LC separation.

2.7. In-solution protein digestion

Seventy micrograms of protein was used for digestion which was performed as described previously [15]. Protein was precipitated by incubation with cold acetone (6 volumes) overnight (−20 °C) and centrifuged at 20,000 × g for 30 min. Pellet samples were then ressuspended with triethyl ammonium bicarbonate buffer (TEAB) (1 M, pH 8.5) and RapiGest (Waters) to a final concentration of 0.5 M and 0.1%, respectively. Samples were reduced with 5 mM tris(2-carboxyethyl) phosphine (TCEP) for 1 h at 37 °C and alkylated with 10 mM S-methyl methanethiosulfonate (MMTS) for 10 min at room temperature. Two micrograms of trypsin were added to each sample and the digestion was performed for 18 h at 37 °C. Samples were dried in a Speed-Vac and then ressupended for peptide separation in high pH reverse phase separation.

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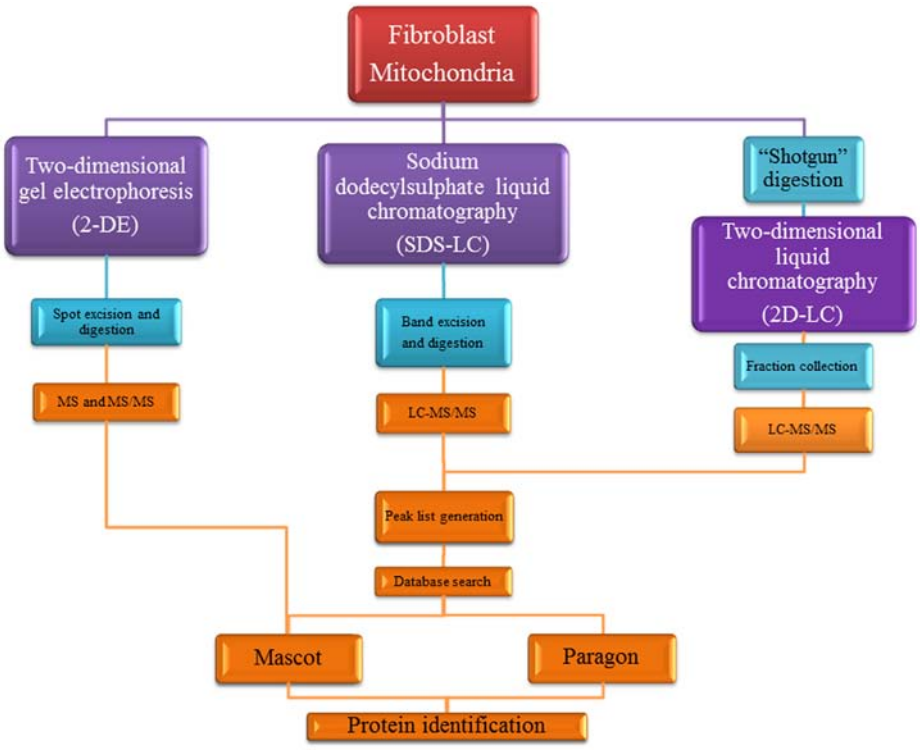

Fig. 1. Schematic flowchart of the methodological strategy used in this study.

2.8. LC analysis

2.8.1. High pH reverse-phase separation

Tryptic peptides from in-solution digestions were ressuspended in buffer A (72 mM triethylamine (TEA), 52 mM acetic acid in H_2O, pH 10). Sample loading was performed at 200 μL/min with buffers A and B (72 mM TEA, 52 mM acetic acid in ACN, pH 10) (98% A: 2% B). After 5 min of sample loading and washing, peptide fractionation was performed in a C18 Acclaim PP2 column (3 μm, 2.1 × 150 mm; Dionex) with linear gradient to 50% B over 35 min followed by a 100% B step. Sixteen fractions were collected, evaporated, and ressuspended in 2% ACN, 0.1% TFA for acidic reverse phase separation.

2.8.2. Reverse-phase separation

Collected tryptic peptide fractions and SDS-PAGE band tryptic peptides were separated as previously described [13]. Briefly, peptides loaded onto a C18 pre-column (5 μm particle size, 5 mm, from Dionex) connected to a PepMap100 C18 (150 mm × 75 μm I.D., 3 μm particle size) column. The flow rate was set at 300 nL/min. The mobile phases A and B were 2% ACN, 0.1% TFA in water and 95% ACN, 0.045% TFA, respectively. The gradient was started at 10 min and ramped to 60% B till 50 min and 100% B at 55 min and retained at 100% B till 65 min. The column was equilibrated with solvent A for 20 min before the next sample was injected. The separation was monitored at 214 nm using a UV detector (Dionex/LC Packings, Sunnyvale, CA) equipped with a 3 nL flow cell. Using the micro-collector Probot (Dionex/LC Packings) and, after a lag time of 5 min, peptides eluting from the capillary column were mixed with a continuous flow of α-CHCA matrix solution (270 nL/min, 2 mg/mL in 70% ACN/0.3% TFA and internal standard Glu-Fib at 15 fmol) were directly deposited onto the LC-MALDI plates at 12 s intervals for each spot (150 nL/fraction). For every separation run, 208 fractions in total were collected.

2.9. Gel-based ID

Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode. Spectra were obtained in the mass range between 700 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, trypsin autolysis, or acrylamide peaks, for subsequent MS/MS data acquisition. Trypsin autolysis peaks were used for internal calibration of the mass spectra, allowing a routine mass accuracy of better than 20 ppm. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot (v2.1.1. Matrix Science Ltd., UK) software for searching the peptide mass fingerprints and MS/MS data. Searches were performed against the Swissprot (release 010111) under all taxonomic categories and the following parameters: (i) two missed cleavages by trypsin; (ii) mass tolerance of precursor ions 25 ppm and product ions 0.3 Da; and (iii) oxidation of methionine and cysteine, phosphorylation (tyrosine, serine and tryptophan) and acetylation (N-terminal and lysine) as variable modifications.

2.10. LC-based ID

Acquisition parameters were similar to Gel-based ID using Glu-Fib for internal calibration. MS/MS data was searched against the Swissprot non-redundant protein database (peptide fragment fingerprinting) (UniProt release 2012-04) with paragon algorithm from ProteinPilot™ software (version 4.0, Applied Biosystems, USA) and Mascot (v2.1.1. Matrix Science Ltd., UK). In order to confidently compare results from Paragon and Mascot, peak lists from 2D-LC and SDS-LC experiments were generated using the Peaks2Mascot (4000 series explorer, Absciex) using the following parameter settings were used: mass range from 65 Da to the precursor mass −35 Da, minimal S/N ratio of 10, minimal area

of 100, maximum of 20 peaks per 100 Da and maximum of 60 peaks per precursor. For Paragon, the search parameters were the following: enzyme (trypsin), special factor (gel-based ID in case of SDS-LC), species (*Homo sapiens*), ID focus (biological modification), detected protein threshold: more than 1 (99%) and running the false discovery rate (FDR). Cut-off score value for accepting protein identification for Paragon® was a ProteoScore of 1.3 (95% confidence). For Mascot (v2.1.1. Matrix Science Ltd., UK), the search parameters were: species (*Homo sapiens*); two missed cleavages by trypsin; mass tolerance of precursor ions 25 ppm and product ions 0.3 Da; and oxidation of methionine, cysteine oxidation (methylthio, in case of SDS-LC), phosphorylation (tyrosine, serine and tryptophan) and acetylation (N-terminal and lysine) as variable modifications. A reverse decoy database was created for all Swissprot to calculate the FDR. Protein identifications were considered reliable when: (i) at least two peptides *per* protein were detected with individual ion score for each peptide greater than or equal to 30; (ii) a single peptide hit with a minimum individual score of 30 (confidence level of at least 95%) and a minimum sequence tag of four amino acids (five consecutive peaks in the MS/MS spectrum).

2.11. Gene network pathway analysis

Gene Ontology (GO) annotations were analyzed with the PANTHER Protein Classification System (database version 6.1, http://www.pantherdb.org/) to identify functional annotations. Protein lists identified by gel- and gel-free methodologies were mapped onto biological pathways that were significantly represented. To evaluate the protein cellular location, the protein lists were analyzed using the ClueGo plugin from cytoscape (v2.8.3) and applying the Gene Ontology database (release date, September 19, 2012). Ontology selection as enrichment analysis was done by right-side hyper-geometric statistic test and its probability value was corrected by the bonferroni method [16].

2.12. Western blotting

Twenty micrograms were subjected to electrophoresis by 15% SDS-PAGE gel, followed by blotting onto a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). After blotting, non-specific binding was blocked with 5% (w/v) non-fat dry milk in TTBS (Tris-buffered saline (TBS) with Tween 20) and the membrane was incubated with primary antibodies diluted 1:1000 in 5% (w/v) non-fat dry milk in TTBS (anti-mitofilin ab48139 and anti-ATPB ab14730 from Abcam; anti-vimentin V6630 from Sigma) for 2 h at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated antibody (GE Healthcare). The blots were developed by using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to manufacturer's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film, Sigma, St. Louis, USA). The protein bands on films were visualized using a GelDoc XR (Bio-Rad) and quantified by Quantity One® Imaging software (v4.6.3, Bio-Rad).

3. Results and discussion

In the present study, a systematic evaluation of mitochondrial proteome was performed using different methodological approaches, gel- and gel free-based. Proteomic analyses were conducted on freshly isolated fibroblast mitochondria from human skin biopsies. The purity of mitochondria was assessed by immunoblotting, which demonstrated the enrichment of mitochondrial proteins (mitofilin, ATP synthase subunit beta) in the mitochondrial fraction isolated from fibroblasts and depletion of the abundant cytosolic protein vimentin (Scheme 1). The yield of isolated mitochondria from this kind of cells is low (approximately 300 μg of mitochondrial proteins per 10^7 cells), which is in accordance with the previously reported by Palmfeldt et al. [1].

Proteomic characterization of isolated mitochondria was performed using three different approaches: 2DE, SDS-LC and 2D-LC, coupled with mass spectrometry for protein identification (Fig. 1). Technical replicates were considered in all the analysis performed (replicates of $n=3$), with an overlapping average of $76 \pm 11\%$ for SDS-LC and 2D-LC. Mascot and Paragon were used for MS/MS processing data. Protein false discovery rates were estimated by the use of reverse decoy sequences during database searching, which were relatively low (≤0.01%). As can be seen in Table 1, a higher number of identified proteins was achieved with SDS-LC approach. Combining data retrieved from all these approaches and search methods, a total of 696 non-redundant proteins were identified, using Swiss-Prot as protein ID search database (Table S1). Based upon the human genome, approximately 2000–2500 mitochondrial proteins are predicted; however, just over 600 have been identified at the protein level (reviewed by [4]). Nonetheless, according to Hermann and Hermann [17], this number can be very variable, ranging from 600 to 4000 proteins. In mitochondria isolated from cultured fibroblasts, more than 1000 distinct proteins were identified using IPI database for protein ID search [1]. The lower number of mitochondrial proteins identified in our study might be justified, at least partially, by the database used for protein ID. Whereas Swiss-Prot database follows a gene-centric approach, with one gene represented by one master protein sequence, IPI database represent a cluster of entries from the source databases and consider splice isoforms as separate entities [18]. Regarding MS/MS processing data with Mascot, an overlap of 14% was observed between the proteins identified by the three different methods (Fig. 2A). When Mascot and Paragon were combined for LC-MS/MS protein identification, this overlap increases to 19% (Fig. 2B). For 2D-LC, 73 unique proteins were identified using Mascot and 136 using Paragon, whereas an overlapping of 42% was noticed (Fig. 2C). In the case of SDS-LC, the same tendency was observed with higher unique proteins identified with Paragon analysis (104 vs. 47). Nevertheless, an overlap of 74% was observed for this methodological approach (Fig. 2D). Using a multidimensional approach combining IEF and LC and comparing with SDS-LC, McDonald et al. [8] only verified an overlap of 9% for inner mitochondria membrane proteins. In the present study, an overlap of 71% was observed when a multidimensional approach combining high pH RP with a standard low pH RP (2D-LC approach) and SDS-LC were used (Fig. 2B), which could be explained by the higher separation efficiency and a more homogenous distribution of eluted peptides [9,19,20]. Interestingly, the intersection between data from the methodological approaches and MS/MS search algorithms for protein ID studied highlights the consistency of 2DE using Mascot in the number of identified proteins. Despite the higher sample amount needed for 2DE, approximately 6-fold greater than the required for

Table 1
Summary of the number of proteins identified by each method and algorithm.

Approach	Proteins identified				Total proteins	
	Replicate A		Replicate B			
	Mascot	ProteinPilot	Mascot	ProteinPilot	Mascot	ProteinPilot
2DE	298	N/A	293	N/A	298	N/A
2D-LC	168	265	172	266	186	284
SDS-LC	417	522	467	513	486	535

SDS-LC and 2D-LC, more distinct proteins were identified using these last two approaches. Indeed, multidimensional chromatographic procedures allow the identification of lower abundant proteins due, at least in part, to the higher resolution underlying the chromatographic separation [19]. However, 2DE allows to distinguish multiple forms of proteins with differences in molecular mass, isoelectric point or PTMs (e.g. phosphorylation) that induces changes in the molecular mass and p*I*, potentially detected

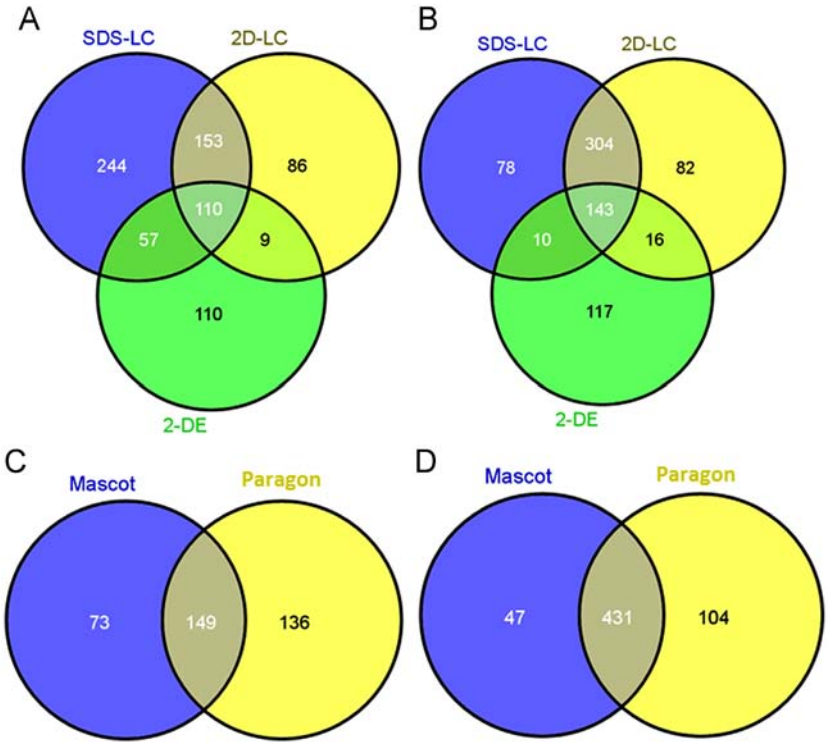

Fig. 2. Venn diagrams indicating the distribution of unique proteins identified using 2D-LC, SDS-LC and 2DE approaches. (A) refers to the distribution of proteins between separation approaches using Mascot. (B) refers to the distribution of proteins between separation approaches combining Mascot and Paragon for SDS-LC and 2D-LC. (C) and (D) refer to the distribution of unique proteins identified with Mascot and Protein Pilot using 2D-LC and SDS-LC, respectively.

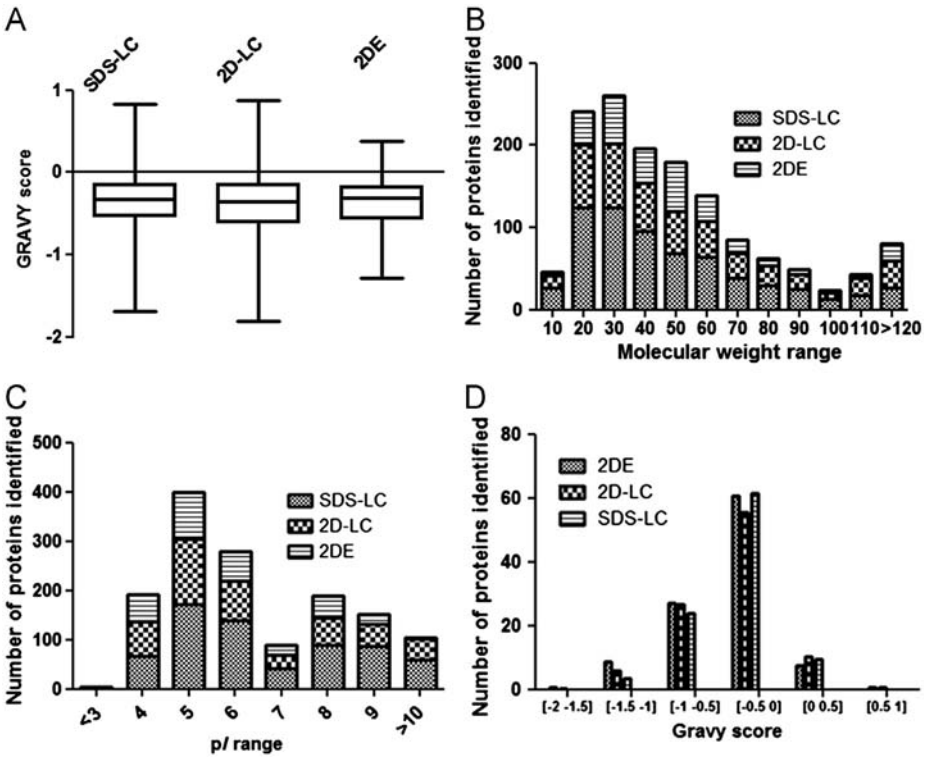

Fig. 3. Gravy score (A), molecular weight (B) and p*I* (C) distribution of identified proteins as a function of protein separation approaches. (D) refers to the Gravy score distribution of unique proteins as a function of separation approaches.

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by deviations in gel's protein migration [21]. In 2DE and SDS-LC some mitochondrial proteins were identified in more than one spot/band (e.g. ATP synthase subunits alpha and beta and aconitate hydratase), evidencing their high turnover [22,23], not distinguished with 2D-LC approach.

In order to search for a potential methodological impact in the intrinsic properties of identified proteins, GRAVY score, molecular weight and isoelectric point were analyzed (Fig. 3). In overall, p*I*, mass and GRAVY score calculations presented a similar tendency among the three methodological approaches used. However, when unique proteins were analyzed in terms of GRAVY score, SDS-LC approach retrieved more hydrophobic proteins (positive GRAVY score) though in relatively lower amount (Fig. 3D). With gel-based approaches involving SDS-PAGE, a significantly higher number of hydrophobic peptides can be recovered [20], which is particularly relevant for mitochondria analysis considering the approximately 33% of membrane proteins (Scheme 2). Even with the optimization of solubilization and running conditions, 2DE does not permit the analysis of proteins with extreme masses (>200 kDa and <10 kDa), hydrophobicity and p*I* [4,21]. 2D-LC and SDS-LC allow the enrichment of proteins with molecular weight below 30 kDa and of basic proteins with p*I* values above 8 (Fig. 3). Independent of the methodological approach chosen, the predominance in mitochondria of hydrophilic proteins (Fig. 3A) with 20–50 kDa (Fig. 3B) and p*I* 5–6 and 8–9 (Fig. 3C) was noticed.

In order to perform cellular localization analysis of all identified proteins, ClueGo [16] was used to determine the association strength between the Gene Ontology (GO) terms. As can be depicted in Scheme 2, most of the identified proteins belong to different organelle compartments related to mitochondria (around 54%). The contribution of ribosomal proteins is also worth of note, justified by the close association between mitochondrial outer membrane and cytosolic ribosomes [24]. Moreover, it has been estimated that approximately 15% of mitochondrial proteins have dual-localization [25]. Regarding biological function, the top three clusters to which these proteins belong are, according to PANTHER bioinformatic tool [26], metabolic processes, cellular processes and transport (Fig. 4) further supported by BiNGO analysis [27] (Scheme 3). Considering the molecular function, the top three clusters retrieved by PANTHER analysis are catalytic activity, structural molecule activity and binding. Despite the similar cluster distribution trend among the three methodological approaches, with 2DE we observed an enrichment of proteins belonging to developmental and cell cycle clusters, and presenting catalytic and antioxidant activity.

In order to get a deep characterization of mitochondria proteome, phosphorylated and acetylated peptides were searched (Fig. 5). These PTMs are known to regulate mitochondrial processes leading to energy production and the synthesis of mitochondrially-encoded subunits of OXPHOS complexes. Though their role in the regulation of cellular events has been known for decades, the extent of this mode of regulation is just beginning to be disclosed for which has contributed the availability of high-resolution mass spectrometers and the development of powerful tools for high scale analysis of PTMs [28,29]. In overall, 101 distinct peptides corresponding to 49 different proteins were found

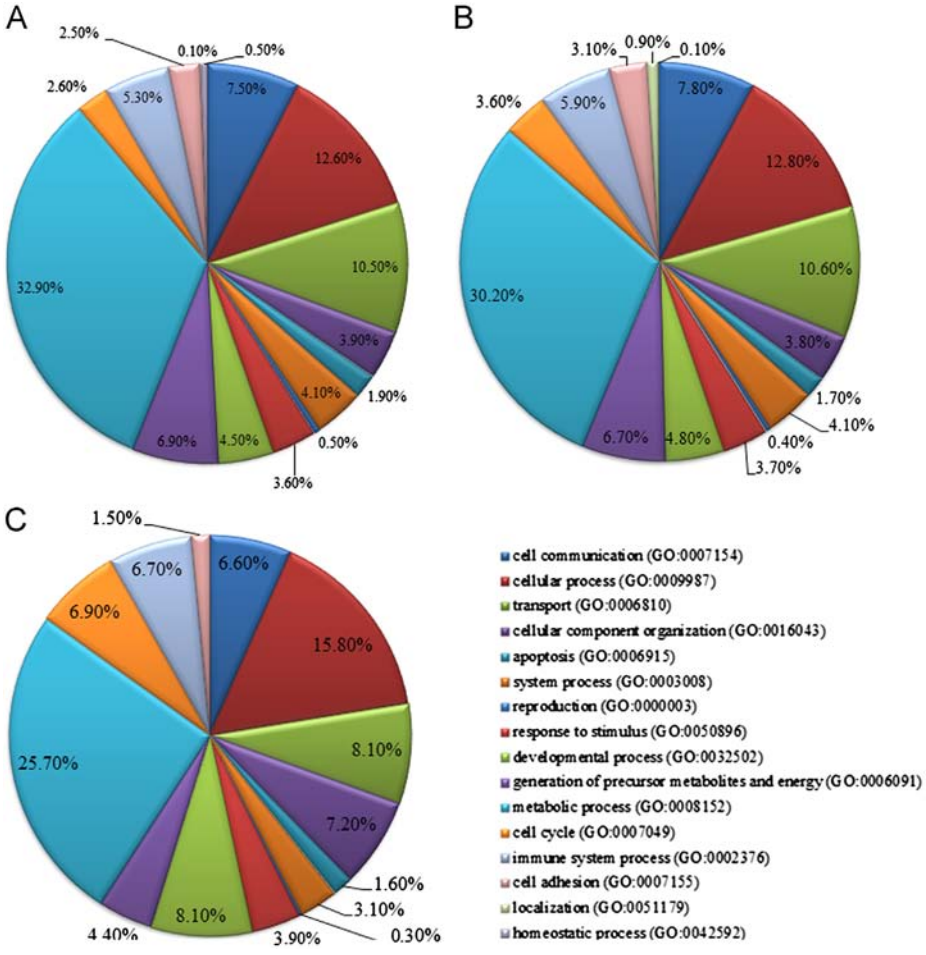

Fig. 4. Pie chart showing the biological function category for the identified proteins using SDS-LC (A), 2D-LC (B) and 2DE (C).

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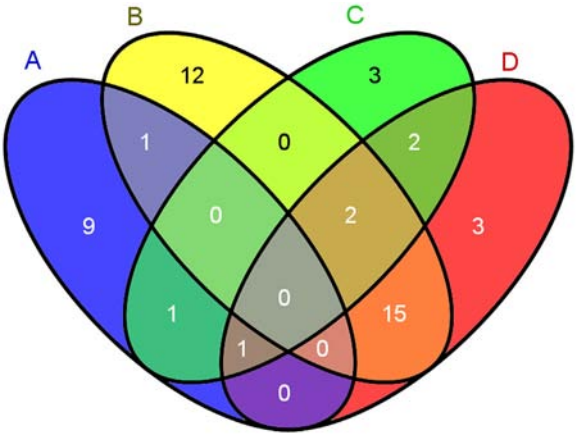

Fig. 5. Venn diagram indicating the distribution of phosphorylated and acetylated proteins using 2D-LC combined with Mascot (A), SDS-LC with Mascot (B), 2D-LC with Paragon (C) and SDS-LC with Paragon (D).

acetylated and phosphorylated. From these, 13 and 30 unique modified proteins were identified by 2D-LC and SDS-LC, respectively. PTMs search with both algorithms produced a similar number of phosphorylated/acetylate peptides identified. Despite the high number of modifications already annotated in Swiss-Prot for the identified mitochondrial proteins, only one peptide from the membrane-associated progesterone receptor component 1 (PGRC1_HUMAN) was found phosphorylated by 2D-LC. Using PTMs Peptide Scanner (v1.0, 〈http://pps.biocuckoo.org/〉), we detected new acetylated sites in the 78 kDa glucose-regulated protein (GRP78_HUMAN; in Lys^{585}), in cytoskeleton-associated protein 4 (CKAP4_HUMAN; in Lys^{551}) and in electron transfer flavoprotein subunit beta (ETFB_HUMAN; in Lys^{201}), using either 2D-LC or SDS-LC and Mascot or Paragon. No enrichment procedures were used for the analysis of acetylation and phosphorylation in mitochondrial proteins, which justify the relatively low number of identified peptides with these PTMs.

4. Conclusion

Data highlight the impact of protein separation methods for protein characterization in mitochondria isolated from cultured fibroblasts. Despite the higher number of identified proteins retrieved by SDS-LC, 2DE allows the identification of a significant content of unique proteins and so might be seen as a complementary approach in a deeper protein profiling of mitochondria. These gel-based approaches are promising when quantitative strategies using metabolic labeling (SILAC) is meant. Nevertheless, 2DE requires a considerable amount of starting protein quantity, which is a limitation for the analysis of mitochondria isolated from samples as cultured fibroblasts. Overall, the results show the power of integrating different separation technologies and protein identification algorithms and suggest caution in making the assumption that similar findings are expected using different separation methods and algorithms.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.026.

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