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Differential liquid phase proteomic analysis of the effect of selenium supplementation in LNCaP cells

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Abstract

The effect of 100 nM sodium selenite supplementation was studied on LNCaP cells by a proteomic approach, on ProteomeLab™ PF 2D platform. Proteins were separated by liquid phase bi-dimensional chromatography and analyzed by pair-wise alignment of peaks to detect those differentially expressed. Differential expression threshold was set at a twice difference level and proteins matching this criterion were identified by MALDI-TOF and confirmed by ESI-ion trap MS/MS. Not all differentially expressed proteins found by PF 2D could be identified by MS analysis, the sensitivity of which emerging as the limiting factor. Thus, only the most abundant proteins, differently expressed following selenium supplementation, were identified. We positively showed an increase of expression of thioredoxin reductase 1, enolase 1, phosphoglycerate mutase 1, glyceraldehyde-3-phosphate dehydrogenase, heterogeneous nuclear ribonucleoprotein A2/B1, isoform A2, Ras-GTPase-activating protein SH3-domain-binding protein and Keratin 18 and a decrease of expression of peroxiredoxin 1 and heat shock protein 70, protein 8, isoform 1. Results are consistent, at least in part, with the less oxidant environment brought about by the synthesis of Se-dependent peroxidases, keeping low the steady-state concentration of hydrogen peroxide.

Keywords: Bi-dimensional chromatography; Selenium; Glycolytic enzymes; PF 2D

1. Introduction

Proteomic analysis provides relevant information about the biological effect of an agonist (e.g. a signaling molecule, drug or nutrient) In the most typical proteomic approach the analysis is carried out by MS identification of proteolytic fragments of proteins appearing differentially expressed by bi-dimensional-electrophoresis [1]. In this study, to take advantage of the good sample capacity and the highly resolved fractionation of multidimensional liquid chromatography [2], we used the ProteomeLab™ PF 2D (Beckman Coulter) platform aiming to detect differentially expressed proteins in cells grown under minimally different culture conditions. As previously reported [3,4], PF 2D fractionates intact proteins by combining a first-dimension chromatofocusing with an on-line second-dimension reverse phase chromatography. Notably, in our hand, a 2D electrophoretic approach failed to detect a different protein pattern under this experimental condition.

We used the prostate cancer cell line LNCaP and the differential challenge was 100 nM sodium selenite. This was suggested by the widely accepted notion that cells in culture, unless specifically supplemented, are partially selenium depleted [5,6].

Selenium is an essential trace element [7], playing its physiological effect when incorporated as selenocysteine into the 25 selenoproteins coded by the human genome i.e. six glutathione peroxidases, three thioredoxin reductases, three thyroid hormone deiodinases, 15 kDa selenoprotein, selenophosphate

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synthase 2, selP, selH, selI, selK, selM, selN, selO, selR, selS, selV and selW [8]. Among these proteins, the enzymes glutathione peroxidases and thioredoxin reductases give an account for the diffused notion of selenium as an antioxidant.

Supplementation is usually carried out with selenium donors, such as selenomethionine or sodium selenite [9], providing the suitable precursor for the co-translational biosynthesis of selenocysteine [10]. Although the expression of selenoenzymes takes place with different saturation kinetics and there is a well-characterized protein hierarchy in selenium incorporation [11], a concentration of 100 nM is seen adequate to guarantee full expression of all selenoproteins in a few days [6].

Selenium is seen as a cancer preventive agent, also independently from supporting selenoprotein synthesis [12]. This has been suggested, indeed, by the possibly relevant role that could be played by some seleno-metabolites [13].

As very little is known about the effect of optimal selenium supplementation on the overall pattern of protein expression, our work was aimed to the analysis of differential protein expression under this condition.

In this work, the proteomic identification of major proteins differentially expressed in the presence of 100 nM sodium selenite is presented following a detailed report of the optimization of the two-dimensional chromatographic approach adopted.

2. Experimental

2.1. Chemicals

Sodium selenite, Tris, Triton-X100, 2-mercaptoethanol and α-cyano-4-hydroxy cinnamic acid (α-HCCA) were from Sigma–Aldrich Inc. (St Louis, MO, US); urea, thiourea, Tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP) and *n*-octyl β-D-glucopyranoside were from AppliChem GmbH (Darmstadt, Germany); acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Romil Ltd. (Cambridge, UK); Criterion™ Precast 12% bis–Tris gels were from Bio-Rad Laboratories, Inc. (Hercules, CA, US); NAP™ 10 and PD 10 desalting columns were purchased from GE Healthcare Europe, GmbH (Freiburg, Germany); ProteomeLab™ PF 2D start buffer and eluent buffer were from Beckman Coulter, Inc. (Fullerton, Ca, US); modified trypsin was purchased from Promega Inc. (Madison, WI, US) and ZipTip$_{C18}$ were purchased from Millipore Co. (Bedford, MA, US).

2.2. Apparatus

2D LC was performed on ProteomeLab™ PF 2D platform (Beckman Coulter, Inc., Fullerton, Ca, US), equipped with an HPCF-1D column (length: 250 mm, internal diameter: 2.1 mm, pore size: 300 Å) and a non-porous (NPS) C_{18} HPRP-2D column (length: 33 mm, internal diameter: 4.6 mm, particle size: 1.5 μm).

Mass spectrometry was performed on a MALDI-TOF mass spectrometer Voyager DE PRO (Applied Biosystems Inc., Foster City, CA, US) and on a LTQ ion trap mass spectrometer equipped with a nano-LC electrospray ionization source (Thermo Finnigan Corp., San Jose, CA, US).

2.3. Cell culture

LNCaP cells were grown as reported [14]. For supplementation experiments, 100 nM Na_2SeO_3 was added to the medium and cells were grown for 3 days before harvesting [14]. Cell pellets were stored at −80 °C until use.

2.4. Activity of selenoperoxidases

Cells from one 75 cm^2 flask were homogenized in 1 ml of 0.1 M Tris–HCl, 0.15 M KCl, 0.05% (v/v) Triton-X100, 5 mM 2-mercaptoethanol, pH 7.8, in a glass/Teflon Potter homogenizer and centrifuged at 105,000 × *g* for 1 h at 4 °C. Supernatants were used for selenoenzyme measurements according to [15].

2.5. 2D LC

2.5.1. Sample preparation

Cell pellets from two 75 cm^2 flasks, at 80% of confluence, were solubilized in 2.5 ml of lysis buffer: 50 mM Tris base, 7 M urea, 2 M thiourea, 4% (w/v) *n*-octyl β-D-glucopyranoside, 10 mM TCEP and a cocktail of protease inhibitors for mammalian cells. Samples were centrifuged at 400,000 × *g* for 30 min at 20 °C. The buffer of solubilized proteins was exchanged using a PD 10 desalting column equilibrated with PF 2D Start Buffer. Before injection, samples were centrifuged at 400,000 × *g* for 30 min at 20 °C.

An equal amount of protein was analyzed for samples to be compared (2.3 mg ± 0.1 mg, Bradford method, BSA as standard).

2.5.2. Chromatofocusing

PF 2D start buffer and PF 2D eluent buffer were carefully adjusted to pH 8.5 with 1.0 M NH_4OH and to pH 4.0 with a saturated solution (50 mg/ml) of iminodiacetic acid, respectively. Chromatographic separation, fraction collection and detection were performed as reported in [3]: briefly, first-dimension chromatofocusing was carried out at room temperature, at a flow rate of 0.2 ml/min. Before injecting the sample, the column was equilibrated with about 50 column volumes until a stable baseline pH (8.5–8.1) was recorded; 20 min after the injection, the elution was started by switching to the eluent buffer at pH 4.0. At the end of this step 1.0 M NaCl eluted proteins still retained on the column. Fractions were collected every 0.3 pH interval during the gradient, otherwise on time basis; in the latter case, 1 ml was the maximum collection volume.

2.5.3. Reverse phase chromatography

Aliquots of 0.2 ml of each chromatofocusing fraction were resolved on the second-dimension chromatography. After a 2-min isocratic step in H_2O–TFA (0.1%, v/v) a 30-min gradient to 100% ACN–TFA (0.08%, v/v) was developed at 50 °C, to improve reproducibility and lower column backpressure. Elution absorbance profile of second-dimension separation was

recorded at 214 nm and fractions were collected every 0.5 min (0.375 ml).

2.5.4. Pair-wise alignments of peaks

Absorbance data from second-dimension chromatographies were used for analysis with ProteoVue™ and DeltaVue™ programs.

Absorbance profiles of fractions of comparable pH (i.e. within 0.15 pH units' difference), obtained from control and selenium treated cells, were matched and picking corresponding peaks generated differential maps and sets of matched chromatographic data.

For differential expression, we used the ratio between the heights of corresponding peaks, after baseline correction. Variation of at least twice as much was considered consistent with a significant difference.

2.6. MALDI-TOF

2.6.1. Sample preparation

Fractions containing differentially expressed peaks were dried in Speed Vac (Savant Instruments Inc., Farmingdale, NY, US) and resolved in 12% bis–Tris gels. After staining with Colloidal Brilliant Blue Coomassie, protein bands were cut and digested overnight at 37 °C with trypsin at enzyme substrate ratio of 1:10 in 40 mM NH_4HCO_3 pH 8.3, 10% (v/v) ACN.

Tryptic peptides were extracted from gel with an equal volume of MilliQ water and ACN–TFA (50%–5%, v/v). Samples were evaporated to dryness, dissolved in water acidified with 0.5% (v/v) TFA and desalted by solid phase extraction, using a $ZipTip_{C18}$. Peptides were eluted in 5 μl of 10 mg/ml α-HCCA in ACN–TFA (50%–0.1%, v/v) and immediately analyzed.

2.6.2. MALDI-TOF conditions and data handling

Mass spectra were acquired operating in the reflectron, delay extraction and positive-ion mode, by accumulating 100 laser shots in an *m/z* range 600–5000 Da.

To optimize mass accuracy an external calibration obtained with peptide standard mixture Calmix2 (Applied Biosystems Inc., Foster City, CA, US) and an internal calibration based on trypsin autolysis peptides, keratin peptides, and blank peptides, applied by Peak Erazor software (version v2.01), were used.

Peptide mass fingerprint analysis was performed on Voyager Data Explorer software (Version 4.0). The resulting peak list was searched against the National Center for Biotechnology Information non-redundant (NCBInr) database (version: 20070120, 20070707, 20070629) by the Mascot interface.

2.7. LC–MS/MS

2.7.1. Sample preparation

PF 2D fractions containing peaks of interest were dried and dissolved in 50 μl of 100 mM ammonium bicarbonate, pH 8.0 and digested by addition of 1 μg of modified trypsin. After overnight incubation at 37 °C the reaction was stopped by acidification with TFA.

2.7.2. LC–MS/MS conditions

Briefly, 5 μl of each digested peptide mixture was directly loaded on a reversed phase column (Biobasic-C18, 0.180 i.d., 100 mm length, 5 μm particle size, Thermo Electron Corp., San Jose, CA, US) and separated with an acetonitrile gradient (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile); the gradient profile was 5% eluent B for 1 min, 5–65% B in 39 min, 65% B for 5 min and 65–85% in 3 min; flow-rate 1 μl/min.

The eluting peptides were electrosprayed directly into a LTQ ion trap mass spectrometer equipped with a nano-LC electrospray ionization source. Full MS spectra were recorded over a 400–2000 *m/z* range followed by five MS/MS events sequentially generated in a data-dependent manner on the first, second, third, fourth and fifth most intense ions selected from the full MS spectrum (at 35% collision energy). Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Finnigan Corp., San Jose, CA, US).

2.7.3. Data handling of MS results

Analysis of MS/MS spectra was carried out using the version 3.2 Bioworks, based on SEQUEST algorithm (University of Washington, licensed to ThermoFinnigan Corp., San Jose, CA, US). The human protein database downloaded from NCBI website (www.ncbi.nlm.nih.gov) in October 2005 was used.

3. Results

LNCaP cells were supplemented with 100 nM sodium selenite for three days, as previously described [13]. In three independent experiments the specific activities of the selenoperoxidases GPx1 and GPx4 increased from 161.0 ± 16.3 to 454.4 ± 35.6 nmol/min/mg and from 46.7 ± 4.5 to 111.7 ± 10.4 nmol/min/mg, respectively.

3.1. Optimization of 2D chromatography

3.1.1. Sample preparation

Ultracentrifugation of the sample, following buffer exchange, was crucial to eliminate traces of particulate material. The procedure had a protein recovery of $88 \pm 6\%$.

3.1.2. Chromatofocusing

Gradient profile was optimized by means of a careful control of pH, temperature of buffers and conditioning of the column. Adjusting the pH of fresh PF 2D eluent buffer, just prior to each chromatography, was also critical.

Reproducibility of chromatofocusing separation resulted in a major problem even when chromatographic conditions were carefully controlled and gradient slope well reproduced. The time between buffer exchange of solubilized proteins in PF 2D start buffer and injection was particularly critical. When sample was injected immediately after the second centrifugation, proteins were optimally distributed along the gradient (Fig. 1A), while, when injection was delayed by just half an hour, they eluted massively in the first fractions (Fig. 1B). As expected, this

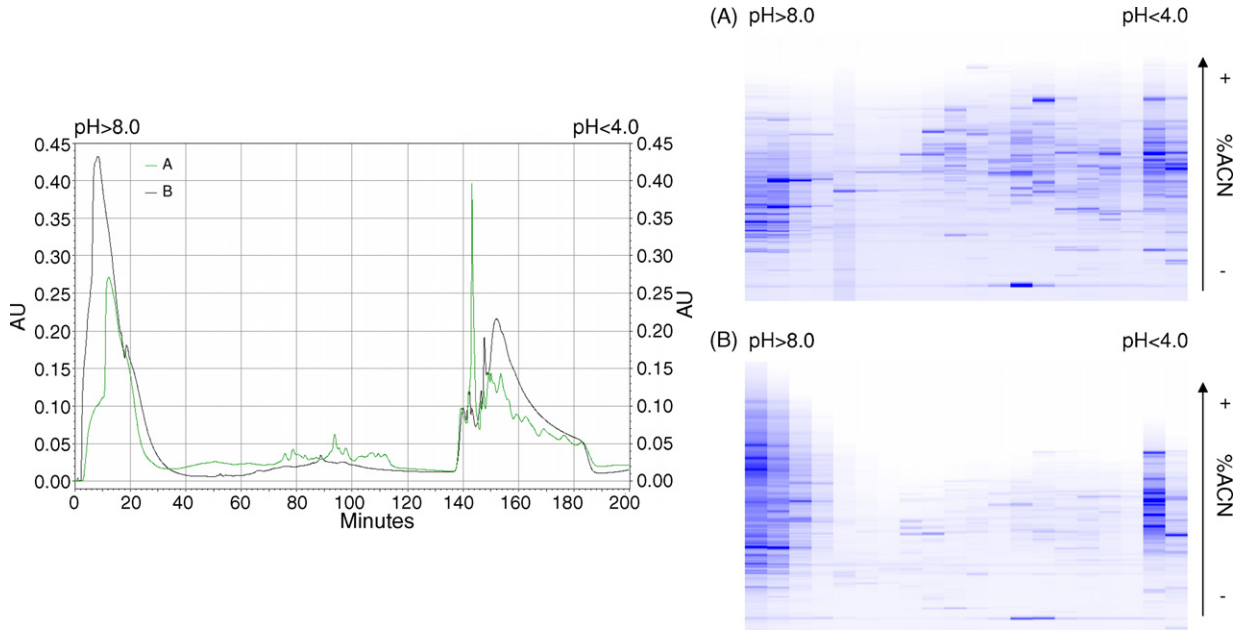

Fig. 1. Effect of delay in sample injection. The panel on the left shows the chromatographic profile of the same sample injected onto chromatofocusing immediately after the last step of sample preparation (green profile) or after a 30 min delay (black profile). An equal amount of protein was used (2.2 mg). On the right the two-dimensional chromatographic maps resulting from second-dimension reverse phase are showed. (A) and (B) are the 2D chromatographic maps corresponding to the green and black profiles of chromatofocusing, respectively. In B basic fractions (pH > 8.0) were dilute 1:1 in PF 2D start buffer to avoid reverse phase column clogging.

artefact was more evident and earlier, when protein concentration was higher.

PF 2D start and eluent buffers are proprietary mixture of urea and *n*-octylglucopyraside. Start buffer contains triethanolamine adjusted to pH 8.5 with saturated iminodiacetic acid and eluent buffer contains ampholytes to pH 4.0. Effective prevention of poor fractionation performance and best reproducibility were obtained by imposing a threshold of protein concentration at 1.5 mg/ml and by respecting the shortest delay between sample preparation and injection.

In attempting to increase the stability of sample, either 0.5 mM DTT or 20% (v/v) isopropanol [16] was added to buffers and sample but this resulted in worst chromatographic resolution. Also the derivatization of thiol groups of solubilized proteins with iodoacetamide before chromatofocusing was ineffective.

It is noteworthy that this problem was particularly evident with LNCaP cells: in fact, so far, this behavior was not so marked with proteins solubilized from other cell lines (e.g. Lx-2, C13) or primary cell cultures (fibroblasts).

Protein recovery from chromatofocusing was $32.2 \pm 3.9\%$.

3.1.3. Reverse phase chromatography

The NPS C_{18} reverse phase chromatography was highly reproducible, retention times generally changing less than 0.05 min for corresponding peaks eluting at the end of the gradient. Moreover, chromatography was optimized in respect to the forthcoming pair-wire analysis, by resolving in sequence corresponding chromatofocusing fractions from samples to be compared.

Basic fractions could cause column clogging, particularly when chromatofocusing fractions contained a relatively larger protein concentration. This problem could be escaped by diluting chromatofocusing fractions that showed an absorbance at 280 nm higher than 0.5 AU. Protein recovery from reverse phase chromatography was $71.8 \pm 5.1\%$.

Fig. 2 reports two examples of proteomic maps: in both samples proteins are evenly distributed along the gradient. A possibly artifactual abundance of proteins is present at pH extremes; anyway, the identity of the vast majority of peaks and the optimal reproducibility guarantees the correctness of comparison between patterns.

3.2. Differential analysis

Differential expression maps between control and selenium treated cells were generated from the 214 nm absorbance profiles of second-dimension runs by using ProteoVue™ and DeltaVue™ programs.

We assessed the reliability of the entire procedure, by comparing repeated injections of the same first-dimension sample at different dilution. Although the variability of the ratio between the heights of corresponding peaks was lower than 15%, for the aim of this study we considered unambiguously valid only differences where the peak height in one sample was at least twice as much than in the other.

From comparative analysis of chromatograms, peaks emerged the content of which were markedly higher in one sample. SDS-PAGE confirmed the difference and provided the sample for MALDI-TOF analysis. As an example, Fig. 3

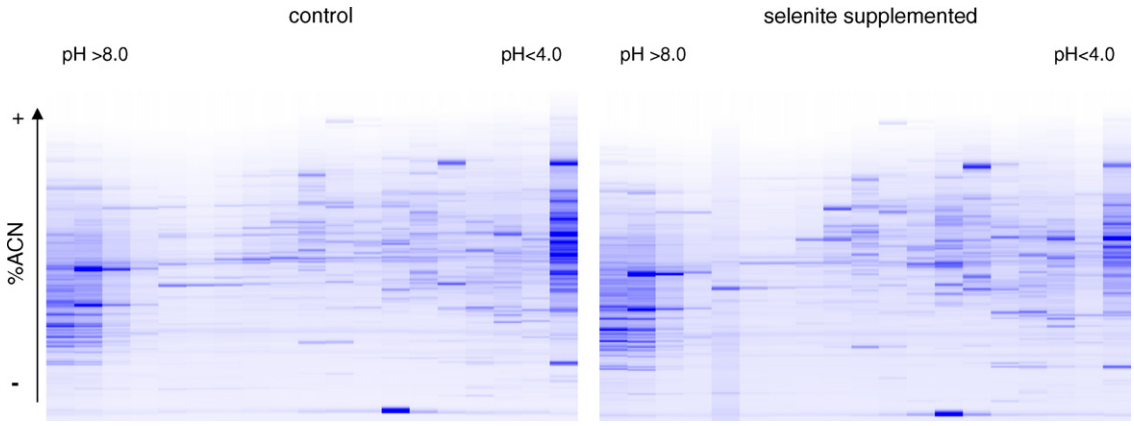

Fig. 2. 2D liquid chromatography separation of intact proteins of LNCaP cells and effect of 100 nM sodium selenite supplementation. Each map was obtained by resolving the same amount of protein and processing of second-dimension absorbance data by ProteoVue™ software. In both 2D chromatography maps there is a rather abundance of bands at the extreme pH lanes. However, this pattern of fractionation is very reproducible and allows easy and reliable comparison of different samples.

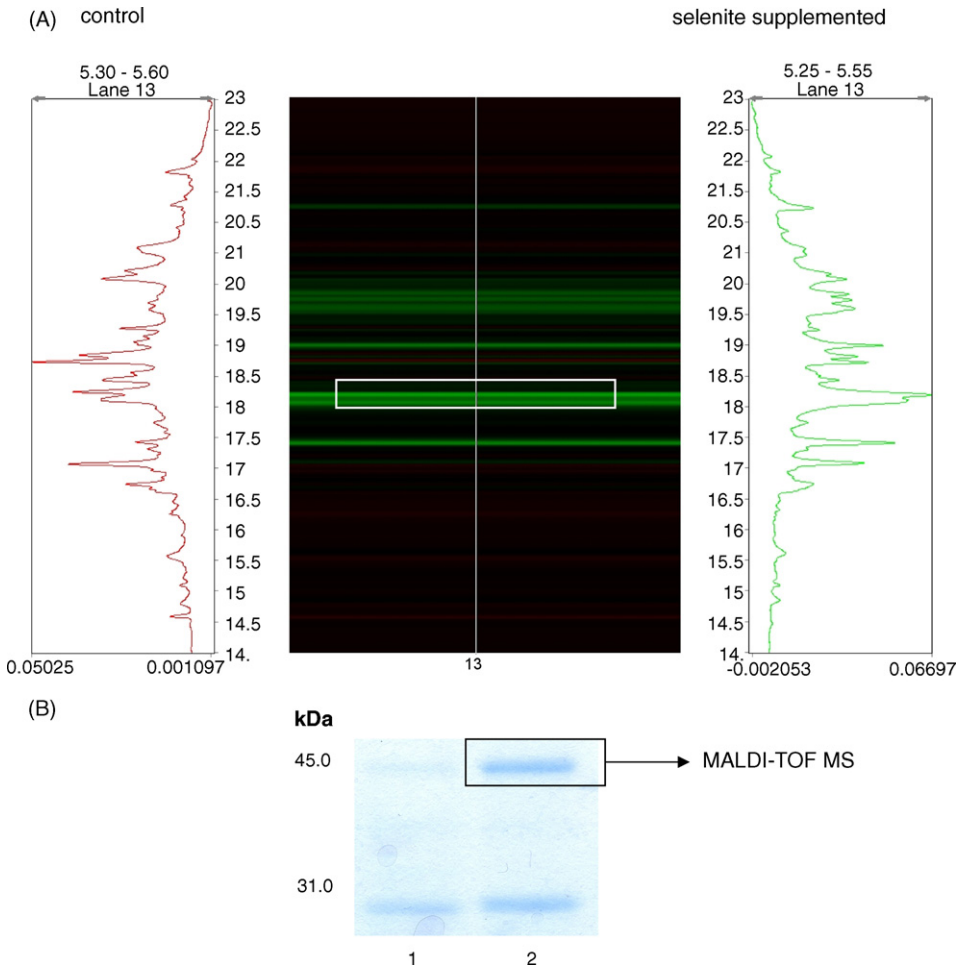

Fig. 3. Identification of enolase 1 as differentially expressed following selenium supplementation. (A) The absorbance traces of reverse phase chromatograms of samples collected within the reported pH intervals are reported. The central panel shows the output of the differential analysis as produced by the DeltaVue™ program. An abundant ($Abs_{214} = 0.58$) differentially expressed ($L/R = 0.478$, where L is control peak height and R is selenium treated peak height) peak (RT = 18.127 min for control and RT = 18.15 min for treated cells) is identified. (B) SDS separation of the fraction identified and collected from both samples, as indicated by the box in the figure. The differentially expressed protein was identified by MS as enolase 1.

shows the identification of a differentially expressed protein (enolase 1).

Since second-dimension samples were collected on time basis, a protein could be eluted in two consecutive fractions. This possible source of misleading results was ruled out by careful comparison of chromatograms and SDS-PAGE pattern of fractions.

In three different experiments, about 750 peaks were detectable in LNCaP cells. According to the imposed threshold for significant difference, $3.1 \pm 0.2\%$ of peaks were over-expressed and $3.4 \pm 0.2\%$ were under-expressed in control cells with respect to 100 nM selenite treated cells. Over-expression or under-expression was always in the range 1:2–1:4.

3.3. Protein identifications

Preliminary experiments determined the absorbance signal intensity threshold for MALDI-TOF analysis. Practically, only peaks high at least 0.04 AU at 214 nm could be further analyzed. This significantly limited the number of samples suitable for MS analysis.

Since more than a single protein could be present in one fraction, SDS-PAGE served as a third-dimension separation to unambiguously select the differentially expressed protein to be identified by MALDI-TOF. This approach gave also useful information about the apparent molecular mass and slightly increased the sensitivity of the overall identification procedure.

The list of differentially expressed proteins following 100 nM selenium supplementation, that could be identified by MALDI-TOF in three independent experiments, both in control and selenium supplemented cells, is reported in Table 1. ESI-ion trap MS/MS confirmed the identifications (Table 2).

These proteins were thioredoxin reductase 1 (TxR1), enolase 1 (ENO1), phosphoglycerate mutase 1 (PGAM1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), heterogeneous nuclear ribonucleoprotein A2/B1 (hNRPA2/B1), isoform A2, Ras-GTPase-activating protein SH3-domain-binding protein (G3BP1) and Keratin 18 (CK18). The missed detection of selenoproteins known to increase under these conditions, such as Se-peroxidases, was somehow expected, due to their relatively low abundance. Among selenoproteins we could detect only thioredoxin reductase 1, this is not surprising as it is often highly expressed in cancer cells [17].

The analysis provided also unambiguous evidence for a drop, brought about by selenium supplementation, of the expression of peroxiredoxin 1 (PRX1) and heat shock protein 70, protein 8, isoform 1 (HSC70).

4. Discussion

In this study, we applied a proteomic approach based on two-dimensional chromatography separation of intact proteins on PF 2D platform, followed by mass spectrometry to identify the major changes of protein expression in LNCaP cells upon supplementation with sodium selenite. The aim was two-fold: critically setting up an accurate proteomic approach in liquid phase, while learning more about the effect of an essential micronutrient.

This multiple chromatographic separation was expected to offer the advantage of higher sample load capacity, so allowing identification of less abundant proteins, and a more straightforward comparison between matched samples for a better reliability of differential quantification.

Sample preparation was accurately standardized. The ultracentrifugation step resulted in fruitful prevention of severe artifacts during the first chromatographic run. Samples proved relatively unstable in PF 2D start buffer, and protein aggregation was observed interfering with chromatofocusing separation. The practical solution was the minimization of the time between buffer exchange and column loading. The protein concentration of the injected sample had to be the best compromise between aggregation prevention and possibility of detection by MS. Nevertheless, despite proper conditions of chromatofocusing, some proteins eluted at pH different from expected (Table 1). This behavior could be suggestive of post-translational modifications, incomplete denaturing of protein complexes or suboptimal chromatographic conditions, as already reported [16].

For differential analysis, second-dimension fractions to be compared were carefully identified on the basis of both pH and absorbance profile of first-dimension separation, as minimal pH shifts, especially at the acidic extreme, could be present. However, the good reproducibility of reverse phase chromatography was useful to recognize minimal shifts in chromatofocusing elution: in fact, peaks with the same retention time in reverse phase separations of adjacent first-dimension fractions, generally proved to be the same protein, as already observed [18]. This was taken into account when defining the differential expression profile.

Data from reverse phase chromatography were analyzed by using the software provided with the PF 2D platform to generate the differential map.

The good reproducibility of two-dimensional protein maps, provided that chromatographic conditions were accurately set up, emerged as the great advantage of this proteomic approach: in fact this allowed the easy and reliable comparison of different samples, not only paired samples (i.e. control and one supplementation condition), but also multiple samples (i.e. control and different supplementation conditions). In the latter case, for repetition of experiments, we used a multiple alignment program specifically developed to this aim [19].

For the present study, fractions containing differently expressed peaks were selected in both samples and resolved in SDS-PAGE. Although not strictly indispensable, this step was introduced since some peaks could contain more than one protein, thus making less certain the identification by MS analysis. Moreover, concentration of the sample increased the sensitivity.

For the final identification of proteins recognized as differently expressed by proteomic analysis, we used a MALDI-TOF instrumentation. However, we also confirmed the identifications produced by peptide mass fingerprint by ESI-ion trap MS/MS. To this purpose, we directly used the sample as produced by chromatography, escaping the SDS-PAGE step. Tandem-MS confirmed the identifications obtained by MALDI-TOF, indi-

Table 1
Effect of sodium selenite supplementation on protein expression in prostate cancer cell line LNCaP

Protein data								Mass fingerprint data			
Protein name	NCBI ID	Selenite	Theoretical p*I*	Experimental pH range	Theoretical MW (kDa)	Experimental MW range (kDa)	Function	Score top match	Score hrnh	Sequence coverage (%)	RMS error (ppm)
Heat shock 70 kDa protein 8, isoform 1	gi|5729877|; ref|NP_006588.1|	Under-expression	5.4	<4.0	70.9	66.2–97.4	Chaperone	105	51	36	14
Peroxiredoxin 1	gi|55959887|; emb|CAI13096.1|	Under-expression	6.4	>8.0	18.9	14.4–21.5	Antioxidant enzyme	70	36	51	26
Thioredoxin reductase 1, isoform 2	gi|33519426|; ref|NP_877393.1|	Over-expression	6.1	>8.0	54.7	45.0–66.2	Antioxidant enzyme	78	64	27	85
Enolase 1	gi|4503571|; ref|NP_001419.1|	Over-expression	7.0	5.3–5.6	47.1	31.0–45.0	Glycolysis, gluconeogenesis	130	53	50	12
Phosphoglycerate; mutase 1	gi|38566176|; gb|AAH62302.1|	Over-expression	6.7	6.5–6.8	28.8	14.4–21.5	Glycolysis, gluconeogenesis	125	41	62	20
Glyceraldehyde-3-phosphate dehydrogenase	gi|89573929|; gb|ABD77190.1|	Over-expression	8.7	>8.0	24.6	31.0–45.0	Glycolysis, gluconeogenesis	72	52	43	21
Heterogeneous nuclear ribonucleoprotein A2/B1, isoform A2	gi|4504447|; ref|NP_002128.1|	Over-expression	8.7	>8.0	36.0	31.0–45.0	RNA metabolism	124	31	45	20
Ras-GTPase-activating protein SH3-domain-binding protein 1	gi|119582066|; gb|EAW61662.1|	Over-expression	5.4	4.1–4.4	52.1	45.0–66.2	RNA metabolism; signaling	70	50	31	47
Keratin 18	gi|4557888|; ref|NP_000215.1|	Over-expression	5.3	5.0–5.3	48.0	45.0–66.2	Cytoskeleton	74	45	33	26

Differentially expressed proteins were identified by peptide mass fingerprint analysis by Mascot interface. For higher stringency in protein identifications, ±100 ppm peptide mass tolerance and 1 possible missed cleavage were allowed. Results were scored using probability-based Mowse Score, where protein scores greater than 65 are statistically significant ($p < 0.05$). For each identified protein accession number, theoretical and experimental p*I* and MW and known biological function are reported. Effects of supplementation are intended for treated cells.
The following mass fingerprint data are also reported: score for identified protein, score for highest ranked non-homologous (hrnh), percentage of protein sequence covered by the matched tryptic fragments and root mean square error.

Table 2
MS/MS identifications for proteins differentially expressed following selenium supplementation

Protein name NCBI ID	Sequence	P(pro)	Protein coverage (%)	X_{corr}	P(pep)	m/z	Charge state
Heat shock 70 kDa, protein 8, gi|48257068|gb|AAH07276.2|		4.66E-09	23.7				
	K.SINPDEAVAYGAAVQAAILSGDK.S			4.81	3.77E-08	2261.47	2
	K.TVTNAVVTVPAYFNDSQR.Q			4.24	8.34E-04	1983.17	2
	K.NQTAEKEEFEHQQK.E			4.03	1.46E-07	1746.82	2
	K.HWPFMVVNDAGRPK.V			3.71	4.66E-09	1654.92	3
	K.SQIHDIVLVGGSTR.I			3.57	4.21E-04	1482.67	2
	K.DAGTIAGLNVLR.I			3.46	5.52E-04	1200.37	2
	K.MKEIAEAYLGK.T			3.46	2.97E-06	1253.49	2
	R.RFDDAVVQSDMK.H			3.25	1.37E-07	1411.57	3
	R.MVNHFIAEFK.R			3.18	8.55E-05	1236.47	2
	K.NSLESYAFNMK.A			3.17	1.03E-03	1304.45	2
Peroxiredoxin 1, gi|55959887|emb|CAI13096.1|		1.92E-06	36.8				
	K.KQGGLGPMNIPLVSDPKR.T			3.01	6.92E-05	1908.26	3
	K.ADEGISFR.G			2.70	2.39E-05	894.95	2
	R.TIAQDYGVLK.A			2.27	1.92E-06	1108.27	2
	R.LVQAFQFTDK.H			2.26	4.02E-03	1197.36	2
	K.IGHPAPNFK.A			2.23	1.17E-04	981.13	2
	K.DISLSDYK.G			1.75	1.33E-03	941.02	1
Alpha enolase, gi|2661039| gb|AAB88178.1|		7.57E-10	54.5				
	R.HIADLAGNSEVILPVPAFNVINGGSHAGNK.L			7.22	7.57E-10	3013.36	3
	K.SFIKDYPVVSIEDPFDQDDWGAWQK.F			5.71	1.88E-10	2987.22	3
	K.FTASAGIQVVGDDLTVTNPK.R			4.69	4.70E-06	2034.26	2
	K.DYPVVSIEDPFDQDDWGAWQK.F			4.53	1.34E-11	2511.64	3
	R.SGKYDLDFKSPDDPSR.Y			4.07	4.26E-07	1827.93	2
	K.DATNVGDEGGFAPNILENK.E			3.90	1.04E-05	1962.06	2
	R.YISPDQLADLYK.S			3.53	1.05E-07	1426.60	2
	R.IGAEVYHNLK.N			3.42	3.70E-04	1144.31	2
	K.VVIGMDVAASEFFR.S			3.25	1.36E-05	1541.80	2
	K.DATNVGDEGGFAPNILENKEGLELLK.T			2.93	2.33E-03	2744.99	3
	K.YDLDFKSPDDPSR.Y			2.67	3.79E-05	1555.63	2
	K.LAQANGWGVMVSHR.S			2.55	1.69E-07	1526.75	2
Phosphoglycerate mutase 1, gi|49456447| emb|CAG46544.1|		1.41E-10	49.6				
	R.RSYDVPPPPMEPDHPFYSNISK.D			5.97	1.41E-10	2574.85	3
	K.HLEGLSEEAIMELNLPTGIPIVYELDK.N			5.41	1.06E-05	3025.46	3
	R.TLWTVLDAIDQMWLPVVR.T			4.07	4.67E-04	2157.56	3
	R.SYDVPPPPMEPDHPFYSNISK.D			3.86	4.97E-04	2418.67	2
	K.NLKPIKPMQFLGDEETVR.K			3.58	1.03E-09	2116.47	3
	R.HGESAWNLENR.F			3.35	4.40E-05	1313.36	2
	R.HYGGLTGLNKAETAAK.H			2.97	5.86E-03	1631.81	3
	R.VLIAAHGNSLR.G			2.40	5.71E-03	1151.34	2

Glyceraldehyde-3-phosphate dehydrogenase, gi|30584593|gb|AAP36549.1|		9.99E-15	38.10				
	K.VIHDNFGIVEGLMTTVHAITATQK.T			6.64	9.99E-15	2596.99	3
	K.VDIVAINDPFIDLNYMVYMFQYDSTHGK.F			5.09	1.26E-09	3310.74	3
	R.VIISAPSADAPMFVMGVNHEK.Y			4.68	6.06E-08	2214.59	2
	K.WGDAGAEYVVESTGVFTTMEK.A			4.38	3.34E-07	2278.48	2
	K.RVIISAPSADAPMFVMGVNHEK.Y			3.85	6.47E-05	2370.78	3
	K.LISWYDNEFGYSNR.V			3.55	5.37E-09	1764.87	2
	K.VGVNGFGR.I			2.39	7.25E-04	805.90	2
Thioredoxin reductase 1, gi|30584095|gb|AAP36296.1|		1.11E-08	11.32				
	K.MIKPFFHSLSEK.Y			3.25	1.11E-08	1464.76	2
Ras-GTPase-activating protein SH3-domain-binding protein, gi|62896771|dbj|BAD96326.1|		5.15E-10	11.8				
	R.HVDAHATLNDGVVVQVMGLLSNNNQALR.R			6.37	2.18E-10	2987.34	3
	K.SSSPAPADIAQTVQEDLR.T			4.50	5.15E-10	1886.01	2
	K.FYVHNDIFR.Y			2.75	3.93E-06	1211.35	2
Keratin 18, gi|12653819|gb|AAH00698.1|		2.11E-14	57.9				
	R.GGMGSGGLATGIAGGLAGMGGIQNEKETMQSLNDR.L			6.96	2.11E-14	3337.71	3
	R.YALQMEQLNGILLHLESELAQTR.A			5.92	9.50E-09	2672.05	3
	R.RLLEDGEDFNLGDALDSSNSMQTIQK.T			5.38	2.25E-07	2898.11	3
	R.PVSSAASVYAGAGGSGSR.I			4.81	3.13E-08	1581.67	2
	K.GLQAQIASSGLTVEVDAPK.S			4.51	1.75E-03	1885.11	2
	R.GGMGSGGLATGIAGGLAGMGGIQNEK.E			4.43	2.65E-06	2262.55	2
	K.NHEEEVKGLQAQIASSGLTVEVDAPK.S			3.97	2.52E-03	2751.00	3
	R.QAQEYEALLNIK.V			3.81	1.16E-03	1420.59	2
	R.SLGSVQAPSYGARPVSSAASVYAGAGGSGSR.I			3.75	4.88E-07	2856.06	3
	R.AQIFANTVDNAR.I			3.71	6.91E-07	1320.44	2
	R.LQLETEIEALKEELLFMK.K			3.62	1.43E-09	2178,57	3
	R.TVQSLEIDLDSMR.N			3.38	2.59E-06	1507.69	2
	K.VKLEAEIATYR.R			3.11	5.34E-05	1293.49	2
	K.YWSQQIEESTTVVTTQSAEVGAAETTLTELRR.T			2.83	3.53E-04	3586.86	3
	R.SLGSVQAPSYGAR.P			2.78	2.23E-05	1293.41	2
	R.KVIDDTNITR.L			2.72	1.27E-05	1175.32	2
	K.LEAEIATYR.R			2.64	1.32E-05	1066.19	2
	K.ETMQSLNDR.L			2.32	2.43E-04	1094.18	2

To increase confidence of protein identification by SEQUEST a great stringency was applied: minimum values of X_{corr} were greater than 1.5, 2.0 and 2.5 for single, double and triple charge ions, respectively, the peptide mass search tolerance was set to 1.0 Da, precursor ion tolerance was set to 1.4 Da, threshold to 100, group scan to 1 Da, minimun group count to 1 and minimun ions count to 15. The stringency applied was optimal as an additional confidence parameter, Δcn (normalized correlation), was better than 0.1 (optimal at >0.07). Moreover, tryptic specificity, two allowed missed cleavages and a tolerance on the mass measurement of 2.0 Da for peptide and 1.0 Da for MS/MS ions were applied. Finally, to assign a final score to proteins, the SEQUEST output data were filtered setting peptide/protein probability to 10^{-3}, consensus score higher than 10 and considering only different peptides. For each identified protein the following data are reported: protein name and NCBI identification number; P(pro), protein probability; protein coverage, percent of protein coverage. For each peptide the following data are reported: Sequence, peptide sequence; X_{corr}, mass spectra correlation; P(pep), peptide probability; m/z and charge state.

cating that, at least under conditions similar to those adopted in this study, MALDI-TOF provides reliable results.

We did not observe any marked difference in the sensitivity between the two MS approaches and in both cases the sensitivity of the MS was always the limit of the overall procedure. Indeed, the number of proteins apparently differentially expressed by liquid phase proteomic analysis exceeded the number of those positively identified by MS.

In this respect, we could conclude that this 2D proteomic chromatographic protocol could be implemented by a more sensitive identification by MS.

Our results, therefore, on differential expression brought about by selenium supplementation had to be concentrated on the most abundant differently expressed proteins.

Differential expression of peroxiredoxin 1 and glyceraldehyde-3-phosphate dehydrogenase was confirmed on cell homogenates by Western blotting, while identical signals were obtained from proteins not identified as differently expressed, i.e. protein disulfide isomerase, heat shock protein 60 and heat shock protein 90, (not shown).

The increase of thioredoxin reductase in selenite-supplemented cells detected in this proteomic analysis, as well as the increase of GPx-1 and GPx-4 enzymatic activity, confirm the notion that cell under usual culture conditions are relatively selenium depleted [5,6].

Proteins under-expressed upon selenite supplementation were peroxiredoxin 1 and HSP70, protein 8, isoform 1 (HSC70). A shift toward a less oxidant environment brought about by increased thioredoxin reductase and glutathione peroxidase activities could likely account for reduced expression of these protein. In fact, as expected under our conditions of selenite supplementation, the concentration of hydrogen peroxide, measured as carboxydichlorofluorescein oxidation rate [20] is decreased approximately by 50% (not shown). HSC70 expression is known to be induced by oxidative stress [21], and a decrease in peroxiredoxin 1 expression by selenium, when selenoperoxidases activity increases, has been already reported on the basis of gene expression profiling [22].

On the other hand, the observed over-expression brought about by selenium supplementation, of a keratin, three glycolytic enzymes and proteins of signaling pathways (heterogeneous nuclear ribonucleoprotein A2/B1, Ras-GTPase-activating protein SH3-domain-binding protein), is more difficult to rationalize on the basis of a different concentration of hydrogen peroxide. It has been reported that selenium activates key proteins involved in the insulin [23] and the epidermal growth factor signal cascade [24]. However, recent evidence suggests that these effects are, at least partially, due to the inhibition of protein tyrosine phosphatases [25], which are inactivated by hydrogen peroxide [26]. This generates a conflict in the straightforward rationalization of the results, since supplementation in selenium-depleted cells, as in our case, actually produces a decrease of hydrogen peroxide. Present evidence, therefore, can just bring a contribution to the description of an extremely complex scenario, where multiple redox transitions take place, within a complex network, the complete rationale of which is still far from being elucidated.

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