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Restricted access chromatographic sample preparation of low mass proteins expressed in human fibroblast cells for proteomics analysis

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Abstract

Two-dimensional electrophoresis and modern image analysis systems have made it possible to study protein expression and regulation of proteins in biological systems. Proteins in the molecular mass region of 20–120 kDa are well investigated and described. However, proteins with masses below 20 kDa are the least investigated as they are rarely seen on 2D-PAGE due to fast migrations in the electric field and lack of staining efficiency. This paper describes a technique that enriches proteins in the lower mass region using solid-phase extraction. The purification step is carried out using C_{18} functionalised ''restricted access'' affinity chromatography whereby simultaneous trace enrichment and sample clean up is achieved. In this study expression patterns of TGF-β stimulated and non-stimulated fibroblasts were compared after the solid-phase fractionation procedure. An increased expression pattern was obtained whereby 400 protein spots could be detected by image analysis in the <20-kDa region. Out of these, specific regulations of 14 spots were found by quantitative image analysis and spots of interest were identified with MALDI TOF–MS. The regulated and identified proteins were triosephosphate isomerase, cofilin and heat shock 27-kDa protein.

Keywords: Proteomics; Two-dimensional gel electrophoresis; Restricted access support; Sample preparation; Proteins

1. Introduction

Proteomics is a research area with fast improvements in methodology as well as new technology introductions [1]. The main interest and focus is set on finding the identities of regulated proteins in biological model systems. The biological material used in these studies are typically linked to a certain disease, a certain biological function, or linked to a signalling pathway [2]. The biological material of choice is in most cases obtained from tissue, cells, or specific compartments in the cells after subcellular fractionations.

The most efficient high resolution separation technique available today for protein separations is still two-dimensional gel electrophoresis [3,4]. However, multidimensional chromatography-based technologies are currently being pursued and developed as a complement to 2D-gel electrophoresis [5–8].

The molecular mass region that is most well described in terms of protein expression and regulation is that of 120–20 kDa. Larger proteins up to around 250 kDa can be determined by homogenous gels (8–10%), by gradient gels, or by crosslinked high resolution one-dimensional gels. The molecular

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mass region that is the least investigated and described is the protein area below 20 kDa. This lower molecular mass region can be expression profiled, but needs specially developed methodologies to reach reproducible differential display of protein expressions in this region. Many important findings can be made in this fractional region such as; chemokines, interleukins and growth factors that are the main regulators of the immune system. Trace enrichment of proteins below 20 kDa can be achieved by applying enrichment of the biomaterial using solid-phase extraction techniques. Specially synthesized micro-extraction supports named ''restricted access'' can be used for this purpose.

The principle of the restricted access support is based upon the presence of two chemically different surface properties of the porous silica particles. The outer surface of the particles (25–40 μm O.D.) are highly biocompatible, it possesses diol modification and is hydrophilic, while the pore surface chemistry is made as a hydrophobic dispersion phase with C_{18} functionality [9].

This selective stationary phase has found applications in a number of areas such as bioanalytical quantification of drugs [9–11], endogenous peptides [12,13], in flow in immunoassay techniques [12–14]. The M_w restriction was found not to be absolute, a smaller part of the pores has an affinity capacity for somewhat larger proteins as well, even human serum albumin was found to have some affinity for the support.

Two-dimensional gel electrophoresis gives a global picture of the protein expression in a cell. However, proteins with low mass, less than 10 kDa, are rarely seen on 2D-PAGE depending on the type of gel used or lack of staining efficiency. In this study we show that with metabolic labeling several new proteins with low masses were obtained. To reveal these proteins on the gel and to identify them by MS analysis they have to be enriched. Here, we describe a solid-phase fractionation procedure that effectively enriches fractions with the low mass range proteins.

In this study we have compared the expression patterns of TGF-β stimulated and non-stimulated fibroblasts in the low mass range of proteins. Several new proteins were revealed and some were regulated in consequence of TGF-β stimulation.

2. Experimental

2.1. Cell culture

The human fibroblasts derived from lung cell line (HFL-1) were obtained from American Tissue Culture Collection (ATCC). The HFL-1 cells were grown in T-75 flasks with MEM medium (Sigma, M-4655) supplemented with 10% FBS and 2 m*M* L-glutamine at 37°C in 5% CO_2 until confluence. The cells were stimulated with 10 ng/ml TGF-β or vehicle for 24 h in same medium as above but with 0.4% FBS. The adherent cells were scraped from the tissue culture flasks, and then harvested by centrifugation (1000 *g*). The cell pellets were stored at −80°C until further use. The amount of cells per T-75 flask was estimated to 10×10^6 cells by cell counting of trypsinated cells.

2.2. Metabolic labeling

Cells were grown as above except that the cells were seeded in a dilution series in 96-well plates starting with 2.5×10^6 cells/ml and 100 μl/well. Cells were stimulated with 10 ng/ml TGF-β or vehicle for 20 h together with 100 μCi ^{35}S-methionine (Amersham Pharmacia Biotech, SJ235)/well in MEM-medium lacking methionine (Sigma, M-3911) supplemented with 0.4% dialysed FCS, 1 m*M* L-glutamine and 0.5 μg/ml cold methionine (Sigma, M-7520). The cells were washed with Hanks buffer and then lysed in 40+80 μl 8 *M* Urea, 2% CHAPS in water.

2.3. Fractionation of samples

The cell pellets were suspended in 1 ml PBS and sonicated for 300 s, with a sequence of 15 s of sonication and 15 s of rest, the output control was set to 2, and kept in ice-water (a Branson Sonifier 250, Branson Ultrasonics Corporation, Danbuty, CT, with a microtip). Insoluble cell debris was removed by centrifugation (10 000 rpm, 5 min, Eppendorf centrifuge). The supernatants were collected and frozen at −80°C until further use.

Fractionation of samples were made using a silica-based restricted access support, ADS C_{18} (kindly provided by Prof. Karl-Siegfried Boos, Clinical Hospital, Gross Hadern Germany) with a particle size of 25–40 μm and pore size of approx. 60 Å. This support was either packed in a column (LiChroCART©25-4, Merck, Darmstadt, Germany) or in a batch procedure in an eppendorf tube. The samples, 25 μl (corresponding to 100 μg protein) or 50 μl (200 μg protein), were diluted in 5% aqueous formic acid to a final volume of 1 ml. All samples were made in triplicates.

The column was washed with 5 ml 75% acetonitrile (ACN), 5% trifluoroacetic acid (TFA) in water and then with 4 ml 1% aqueous TFA. The sample, 1 ml, was gently pushed, by using a syringe, through the column and the flow through fraction was collected. The column was washed with 3 ml 1% aqueous TFA. Elution of desorb material was made by 2 ml 60% ACN, 0.1% TFA in water. Before next sample were applied, the column was washed with 1 ml 75% ACN, 5% TFA in water followed by 4 ml 1% aqueous TFA.

In the batch procedure, approximately 20 mg of ADS C_{18} was washed with 1 ml 75% ACN, 5% TFA in water followed by centrifugation. Another wash with 1 ml 1% aqueous TFA. The sample, 1 ml, was mixed with the solid phase for 20 min on a shake table. The mixture was centrifuged and the supernatant was collected. The solid-phase was washed with 1 ml 1% aqueous TFA by shaking and by subsequent centrifugation. The elution was made by mixing the solid phase with 60% ACN, 0.1% TFA in water followed by centrifugation and the supernatant was collected. All collected fractions were evaporated in a Speed Vac to complete dryness. The residue was solved in 100 μl of 7 *M* thiourea, 2 *M* urea, 4% (w/v) CHAPS in water. The samples were frozen at −80°C until further use.

2.4. Protein determination

Protein was determined by Bio-Rad protein assay (Cat. No. 500-0116, Bio-Rad Laboratories, Sundbyberg, Sweden) for microtiter plate and with bovine serum albumin as standard.

2.5. One-dimensional gel electrophoresis

One-dimensional gel electrophoresis was performed using precasted 4–12% NuPAGE (8×8 cm) gels from Novex. The fractionated sample, 10 μl, were mixed with 10 μl sample buffer (NuPAGE Sample buffer NP0003, Novex, San Diego, CA, USA). Electrophoresis (200 V constant voltage) was carried out in a Xell™ Mini-Cell apparatus (Novex, San Diego, CA, USA) for about 45 min.

2.6. Two-dimensional gel electrophoresis

Immobiline Dry strips (180×3×0.5 mm, pH 3–10 NL) were rehydrated in 350 μl of the solubilisation solution containing 7 *M* thiourea, 2 *M* urea, 4% CHAPS, 10 m*M* DTT, and 0.5% IPG 3–10 buffer, together with the fractionated samples (100 μl) or the same amount of radioactivity for the metabolic labeled samples.

The isoelectrophofocusing (IEF) step was performed at 20°C in a IPGphor™ (Amersham Pharmacia Biotech, Uppsala, Sweden) and run according to the following schedule: (1) 30 V for 10 h, (2) 500 V for 1 h, (3) 1000 V for 1 h, and (4) 4000 V until approximately 45 000 Vh were reached. The strips were equilibrated for 10 min in a solution containing 65 m*M* DTT, 6 *M* urea, 30% (w/v) glycerol, 2% (w/v) SDS and 50 m*M* Tris–HCl pH 8.8. A second equilibration step was also carried out for 10 min in the same solution except for DTT, which was replaced by 259 m*M* iodoacetamide. The strips were soaked in electrophoresis buffer (24 m*M* Tris base, 0.2 *M* Glycine and 0.1% SDS) just before the second-dimensional gel electrophoresis. The strips were applied on 14% homogeneous Duracryl slabgel. The strips were overlaid with a solution of 1% agarose in electrophoresis buffer (kept at 60°C). Electrophoresis was carried out in an Hoefer™ DALT gel apparatus (Amersham Pharmacia Biotech, San Francisco, CA, USA) at 20°C and constant 100 V for 18 h.

For mini gel separation, Immobiline Dry strips (70×3×0.5 mm, pH 3–10 NL) were used with same solubilisation conditions as above. Electrophoresis in the second dimension was carried out in a XCell II

Mini-Cell (Novex, San Diego, CA, USA) at 125 V for 2 h and 15 min.

2.7. Gel staining

Gels were stained with silver according to Shevchenko [15] or stained with SyproRuby (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendations, or by ^{35}S-methionine.

2.8. Spot analysis

Gels were scanned using a Fluor-S™ MultiImager (Bio-Rad Laboratories, Sundbyberg, Sweden) and Quantity One (version 4.0.3, Bio-Rad Laboratories, Sundbyberg, Sweden). Clear filter, white light, transillumination, high resolution, aperture 5.6 and 300 mm scan were parameters selected for the scanning of the silver stained gels. For gels stained with SyproRuby the settings were 520LP filter, UV-light, Epi, high resolution, aperture 2.7 and 300 mm scan. Spot analysis was performed using the PDQUEST (version 6.1.0) two-dimensional gel analysis system (Bio-Rad discovery series, Bio-Rad Laboratories, Sundbyberg, Sweden).

The ^{35}S-methionine labeled gels were scanned using a phosphorImager (STORM 860, Molecular Dynamics, Sunnyvale, CA, USA).

2.9. Mass spectrometry identification

The MALDI–TOF instrument was a Voyager DE-PRO (Perseptive Biosystems Inc., Framingham, MA, USA) mass spectrometer. The instrument, equipped with a delayed extraction ion source, utilize a nitrogen laser at 337 nm and was operated in reflectron mode at an accelerating voltage of 20 kV. The sample probes were made of polished stainless steel. Sample deposition of nanoliter fractions was made on stainless steel MALDI-target plates and on a 400-position Teflon target plate (Perseptive Biosystems). The nanoliter fractions were gained by manually eluting small droplets from microextraction columns onto the MALDI target plate according to [16].

Gel extraction prior to MALDI identifications were made according to Shevchenko et al. [15].

3. Results and discussion

Selective sample handling reaching enrichment factors of solubilised proteins from fibroblasts can be achieved by using a selective extracting support material such as ''restricted access'' affinity chromatography. With a defined pore size, proteins with a molecular mass region of choice can be enriched whereby discrimination is achieved towards larger sized proteins using the ''restricted access'' silica material. The outer surface of these packings possesses a defined diffusion barrier and a non-adsorptive outer particle surface towards macromolecular matrix proteins larger than 15 kDa [9–11]. Small molecular mass proteins $<$15 kDa will diffuse into these freely accessible affinity centers, i.e. the pores (approximately 60 Å) and be bound to the hydrophobic C_{18} groups, this part of the binding mechanism forms the physical diffusion barrier [11].

In order to enrich fractions with low masses and also to eliminate proteins high molecular masses, a solid-phase extraction methodology and protocols were established according to the scheme shown in Fig. 1. After cells were grown to confluence, the cytokine growth factor TGF-β was added to trigger the cells. After homogenisation and centrifugation,

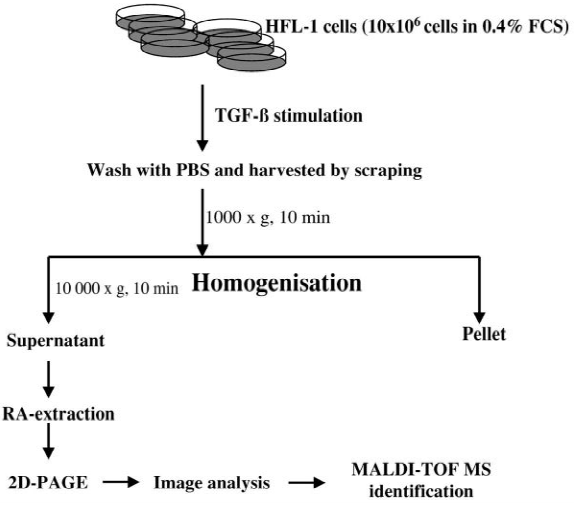


Fig. 1. Flow chart summarising the solid-phase fraction procedure from the cultured and stimulated fibroblasts to an enrichment and sample clean up.

the resulting soluble fraction of the fibroblasts was amenable for sample preparation.

Silver and fluorescence staining of HFL-1 reveals similar protein expression patterns (see Fig. 2a and b). About 1000 spots can be detected by image analysis using the PDQUEST imaging software using these two stains. ^{35}S-methionine metabolic labeling increases the possibility to visualize newly synthesized proteins, whereby 3500–4000 spots can be made visual (see Fig. 2c). From these experiments it was clear that the expression profiles could be increased by making HFL-1 enrichment utilizing the restricted access extraction technique. It should be emphasized though that the metabolic labeling images are not directly comparable with the silver and fluorescent stained gels due to the differences taken in the two experimental approaches. The staining in Fig. 2a and b being post gel image read-outs while the labeling is an isotope incorporation that displays the ^{35}S-methionine that is used as the amino acid upon protein synthesis. Nevertheless, the improvement of visualization of the <20 kDa region of TGF-β stimulated human fibroblasts is made clear. The metabolic labeling of fibroblasts also revealed a number of new proteins with low masses not seen with silver staining or SyproRuby (see Fig. 2).

The capacity of the extraction material has been determined previously [13] for low-molecular mass peptide leukotrienes. We have made capacity experiments where we were able to run peptide levels of 60 μg/50 mg support without any breakthrough. The adsorption and desorption kinetics were investigated by using Lysozyme, ribonuclease A and Cytochrome C. It was found that distinct adsorption is obtained in aqueous eluents such as PBS or acidic buffers such as TFA or formic acid. Step gradient desorption is achieved by using acetonitrile/water (50:50% v/v) as a desorbing agent.

Traditional enrichment of proteins by precipitation was found not to be an option for proteins with low masses. Fewer proteins were found to be displayed on the 2D-PAGE. (data not shown). It seems that these proteins have better solubility in the organic/aqueous precipitation agents. Sample preparation of solubilised proteins derived from the soluble protein fraction of HFL-1 was performed using the restricted access biocompatible <20 kDa protein enrichment. The 1D-gel (Fig. 3a) of fractions from the solid-phase extraction showed that there were an enrichment of proteins with low masses in the eluted fraction (Fig. 3a, lane 3) and proteins with masses over 40 kDa were in the flow through fraction (Fig.

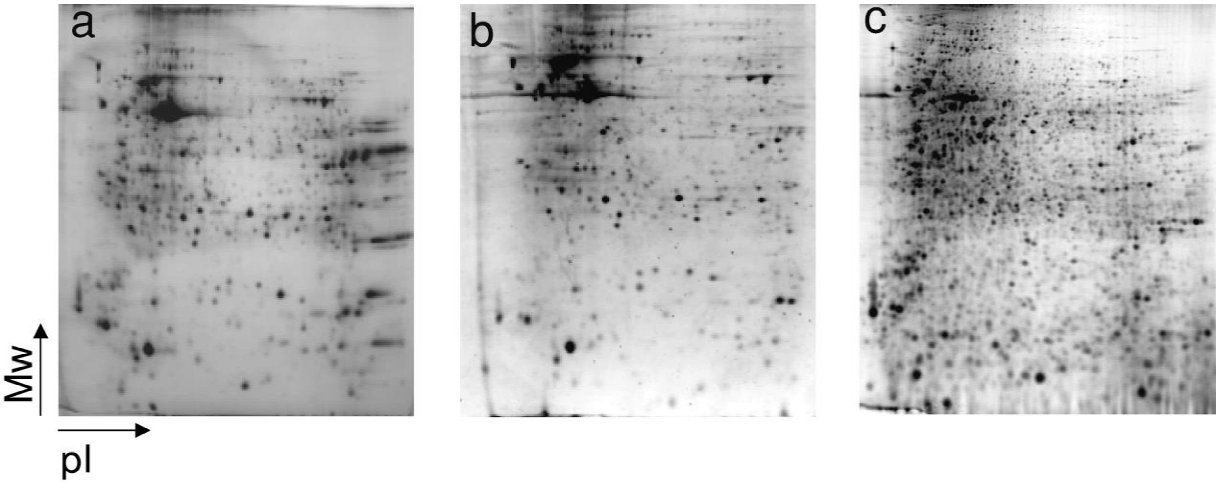


Fig. 2. 2D-PAGE of HFL-1 cells stimulated with 10 ng TGF-b/ml for 20 h. Proteins were visualised by: (a) silver and (b) SyproRuby; and (c) proteins were labeled with ^{35}S-methionine. Isoelectric focusing in the first dimension was carried out on non-linear pH 3–10; 18-cm immobilised pH gradients strips. The load was 100 μg of total cellular protein for (a) and (b) and approximately 15 μg for (c). For the second dimension, 14% Duracryl gels were used.

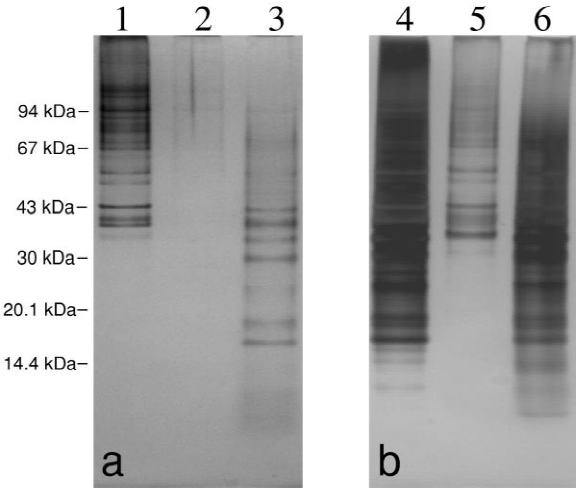


Fig. 3. 1D-PAGE (4–12% NuPAGE with MES as running buffer) of fractions from the solid-phase extraction, flow through (lanes 1, 4), wash fraction (lane 2, 5) and eluted fraction (lane 3, 6). To the column: (a) 100 μg and (b) 200 μg protein of TGF-b stimulated HFL-1 cells was loaded.

3a, lane 1). The column was washed before the desorption fraction was eluted with 60% ACN, 0.1% TFA in water. This wash fraction (Fig. 3a, lane 2) show that only proteins with masses above 40 kDa are washed out and those proteins with masses below 40 kDa is well adsorbed to the column.

However, overloading the column resulted in poor separation (Fig. 3b). There is not that much of a difference between the load fraction (Fig. 3b, lane 4) and the eluted fraction (Fig. 3b, lane 6). Although, the wash fraction (Fig. 3b, lane 5) only eliminates proteins with masses over 40 kDa.

Solid-phase extraction was performed and compared between column and batch methodologies. The batch mode of operation was performed by using 20-mg support material in 1.5 ml tubes. Sample deposition, washing and desorption was made by careful pipetting the supernatant from the support pellet in the test tubes after centrifugation steps. With the column procedure there was a clear cut-off regarding the masses, which was not obtained by the batch procedure (Fig. 4). The column procedure removed high molecular mass proteins, which were found in the flow through fraction (Fig. 4a). Proteins with masses mainly below 40 kDa were found in the eluted fraction (Fig. 4b). In the batch mode the separation of proteins were not at all distinct. Both the flow through (Fig. 4c) and the eluted fraction (Fig. 4d) revealed similar 2D-PAGE protein expression pattern. These advantages made us use the column methodology.

By the enrichment using solid-phase extraction, fibroblast stimulated with TGF-β revealed new proteins of low masses that were regulated. The protein extraction procedure was more efficient and therefore more proteins could be detected. This is most noteworthy in the neutral pI-region. Specific regulations of 14 spots could be identified by image analysis in the lower M_w region.

These specific and significant regulations in fibroblasts, triggered by TGF-β were found in the molecular mass region 6–27 kDa. Most of these findings were not detectable by using traditional proteomics physio-chemical sample preparation methodology in combination with 2D-gels. These findings even strengthens the value of using the restricted access solid-phase extraction technique.

The difference in expression profiles is not strikingly obvious by just looking at the gel images. However, by software spot imaging, we find that 200 weaker spots appear from the enriched cell supernatants on the gel. Applying the same imaging on untreated (without performing clean-up experiments) cell supernatant, these 200 spots are not detectable on the silver stained gel.

In the mass range of 50–18 kDa, 14 spots were identified by MALDI TOF–MS. The identities of these proteins are marked with their names in Fig. 5. After TGF-β stimulation we found that three out of these identified spots in the low mass region were regulated. Additionally, 11 proteins were regulated but not identified and are only marked with their respective spot number. Actin, the vimentin isoforms, tropomyosin and β-tubulin, are house keeping proteins of the HFL-1 cells and are marked on the Ref. gel in Fig. 5. The regulated and identified proteins were identified as triosephosphate isomerase, cofilin, and heat shock 27-kDa protein (HSP27). The triosephosphate isomerase plays an important role in several metabolic pathways such as glycolysis and glycerolipid metabolism. Triosephosphate isomerase deficiency is the most severe clinical disorder of the glycolysis. It is associated with neonatal jaundice, chronic hemolytic anemia, progressive neuromuscular dysfunction, cardiomyopathy, and

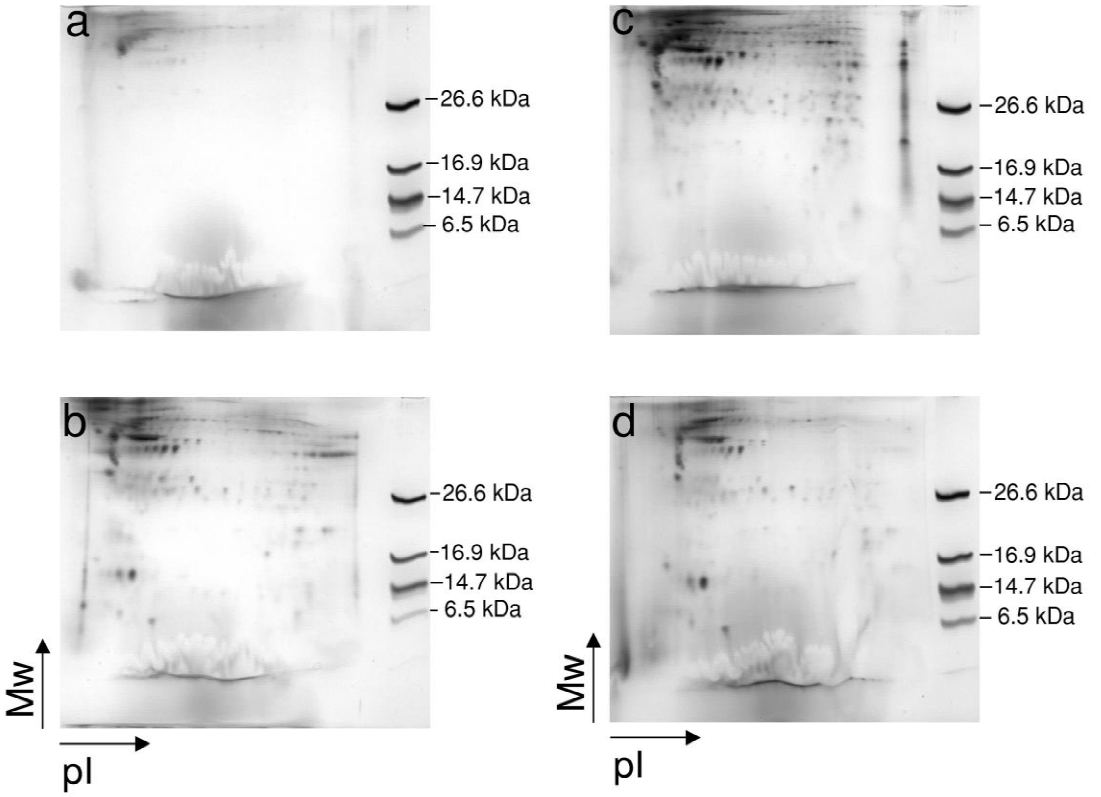


Fig. 4. 2D-PAGE of fraction from solid-phase extraction by column (a and b) and batch procedure (c and d). Flow through fractions represents by (a) and (c) and eluted fractions by (b) and (d). Isoelectric focusing in the first dimension was carried out on non-linear pH 3–10; 7 cm immobilised pH gradients strips. For the second dimension, 18% Duracryl gels were used.

increased susceptibility to infection [17–19]. Cofilin controls reversibly actin polymerization and depolymerization. HSP27 belongs to a large group of polypeptides, the stress proteins that are induced in various combinations in response to environmental challenges and development transitions. HSP27 is involved in stress resistance and actin organization [20–23]. It is also one protein in the family that is involved mechanistically as the substrate for a number of kinases [24,25], also shows the importance identifying this protein expressed in fibroblasts. The resulting peptide map fingerprint identified by MALDI–TOF MS is depicted in Fig. 6. The spectrum was internally calibrated using the trypsin autodigest peaks at m/z 842.510 and m/z 2211.105, affording high mass accuracy (delta value <15 ppm). Six peptides could be matched with a sequence coverage of the protein of 34%.

Interestingly, making a sequence alignment in the Swissprot data base all the human heat shock proteins found, showed only minor similarities between HSP 27 and other members of the Heat Shock Protein family. With an additional BLAST-search using the tryptic peptides of HSP 27 (indicated in Fig. 6) versus the Swissprot resulted in HSP 27 as the only human heat shock protein that matched the sequences in the data base. Finally, these MALDI peptide sequences assures the identity of HSP 27.

4. Conclusions

A solid-phase extraction methodology involving C_{18} functionalised ‘‘restricted access’’ affinity chromatography has been established in this work. The

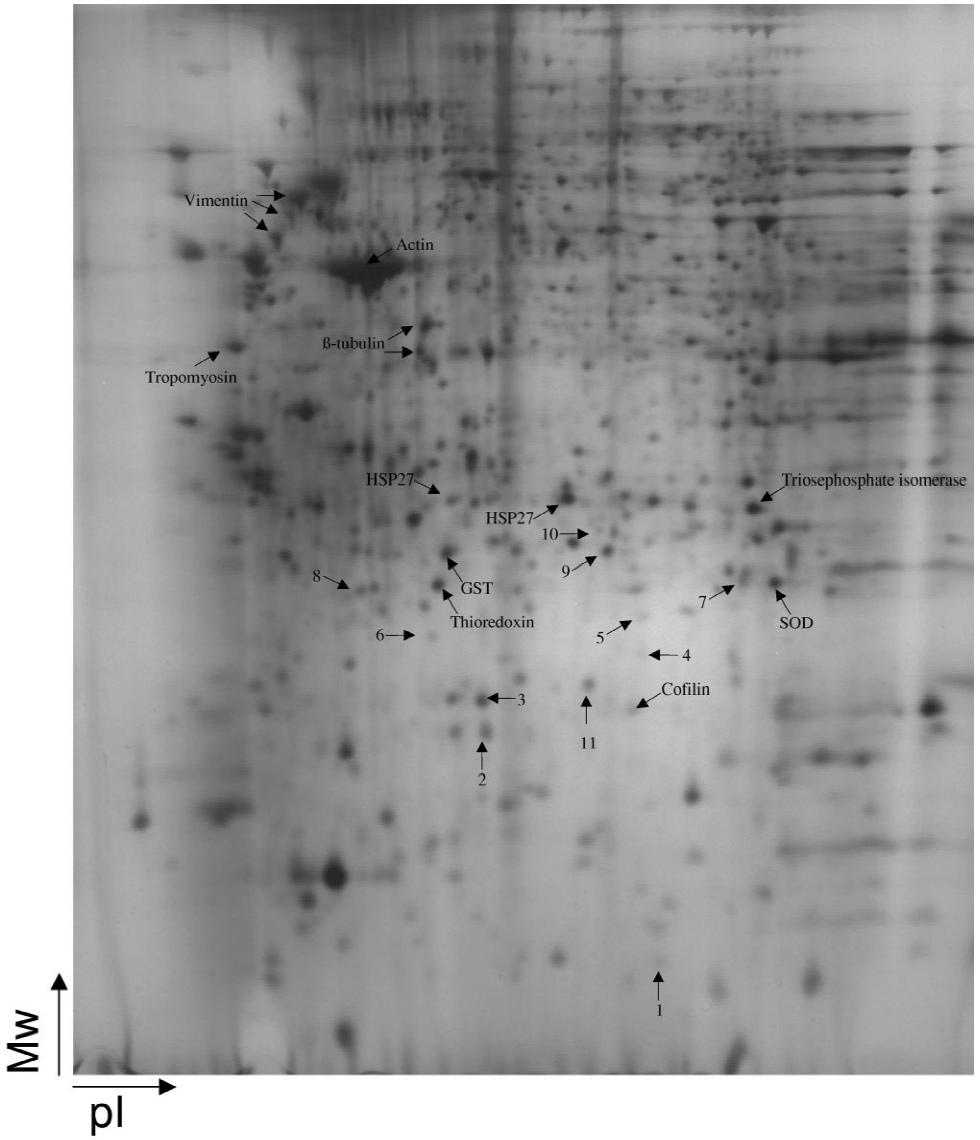
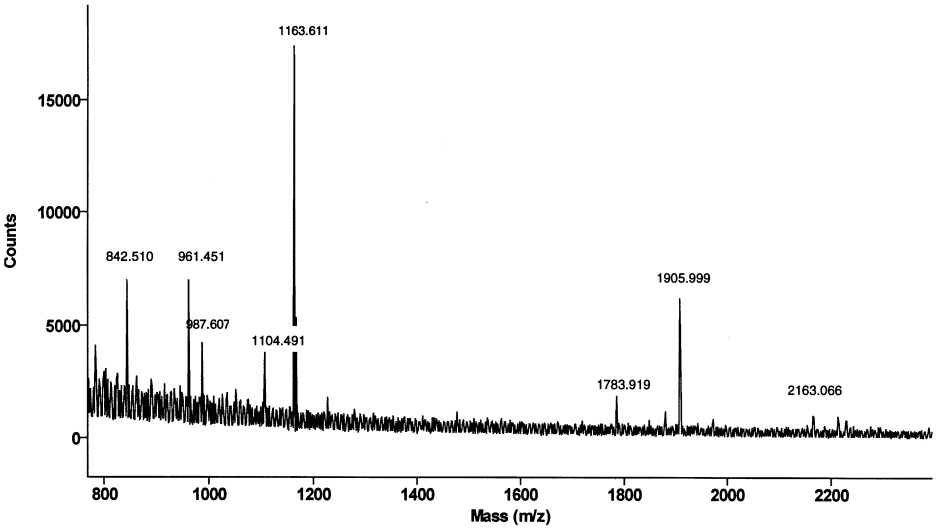


Fig. 5. 2D-PAGE of a fraction enriched with solid-phase extraction. Structure proteins and three of the regulated proteins in consequence of TGF-b stimulation are marked with their names. Other regulated proteins are only marked with spot number. Isoelectric focusing in the first dimension was carried out on non-linear pH 3–10; 18-cm immobilised pH gradients strips. For the second dimension, 14% Duracryl gels were used.

use of this extraction method made it possible study enriched protein fraction with low masses from HFL-1 cells and their changes in protein expression in consequence of TGF-β stimulation. The pre-extraction increased the overall protein expression of the HFL-1 cells revealed on the 2D-PAGE especially in the neutral pI-region. Specific regulations were found for 14 spots in the low mass range and three was identified with MALDI TOF–MS. The method described here will be useful in proteomics work for enrichment and identification of proteins in the lower mass region from biological samples such as solubil-



1. P04792 **Mass:** 22427 **Score:** 92
heat shock 27 kd protein (hsp 27) (stress-responsilhomo sapiens)

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
961.451	960.443	960.449	- 6ppm	13	20	0	GPSWDPFR
987.607	986.599	986.602	-3ppm	5	12	1	RVPFSLLR
1104.491	1103.483	1103.500	-15ppm	128	136	0	QDEHGYISR
1163.611	1162.604	1162.613	-8ppm	28	37	0	LFDQAFGLPR
1783.919	1782.911	1782.918	-4ppm	97	112	0	VSLDVNHFAPDELTVK
1905.999	1904.991	1904.984	4ppm	172	188	0	LATQSNEITIPVTFESR

Fig. 6. MALDI mass spectrum of heat shock 27-kDa protein. Six peaks of this spectrum whose mass was detected matched the peptide sequence and covered 34% of the protein.

ised cells, bronchoalveolar lavage fluids and synovial fluids.

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