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Fractionation of liver proteins by preparative electrophoresis

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Summary. Proteomics offers unique possibilities to investigate changes in the levels and modifications of proteins involved in the pathomechanisms of diseases and toxic events. However, search for potential drug targets and disease or toxicity markers is limited by the fact that mainly the high-abundance, hydrophilic proteins are visualized in two-dimensional gels. Here we studied the enrichment of rat liver cytosolic proteins by preparative electrophoresis. Preparative electrophoresis was performed with the PrepCell apparatus in the presence of 0.1% lithium dodecyl sulfate. Lithium dodecyl sulfate was exchanged against agents compatible with isoelectric focusing prior to the two-dimensional gel electrophoresis. Proteins were identified from two-dimensional gels by matrix-assisted laser desorption ionization time-of-flight mass specrometry. Low- and middle-size proteins and low-abundance proteins, which had not been found before, were enriched by preparative electrophoresis. The present study represents a contribution of proteomics in the quantification of differences in the levels of low-abundance liver proteins in toxicity studies.

Keywords: Proteomics – Preparative electrophoresis – Liver – Low-abundance proteins – Mass spectrometry

Introduction

Proteomics is frequently applied in the investigation of toxic events because it enables the efficient generation of toxicity-related protein patterns, which may be useful in predicting toxicity of drug candidates (Steiner and Witzmann, 2000). Proteomics usually involves protein separation by two-dimensional electrophoresis and protein identification mainly by mass spectrometry (Fountoulakis, 2001). The performance of the proteomics technologies has been greatly improved in the last few years. The technology currently allows the identification of all gene products of a proteome, which are expressed in sufficient amounts to be visualized in gels stained with Coomassie blue. However, there is still a large discrepancy between possible and detected gene products in a proteome. To increase the likelihood of detection of low-abundance proteins in complex biological mixtures, the proteomic analysis is increasingly directed to simpler protein fractions, each containing a lower number of components in comparison with the starting material (Fountoulakis and Takács, 1998). Investigating biological events, it is interesting to study low-copy-number gene products because such proteins are most likely the potential drug targets or markers related to toxicity pathways.

The separation of a protein mixture into organelle fractions prior to the 2-D electrophoresis analysis is usually the first step to increase the probability of detecting low-copy-number gene products. Subsequent enrichment of proteins from larger volumes is usually achieved by selective fractionation, chromatography or electrophoretic procedures (Fountoulakis and Takács, 2002). The electrophoretic methods comprise the separation of protein mixtures by preparative polyacrylamide gel electrophoresis on the basis of protein size usually in the presence of ionic detergents, or by preparative isoelectrofocusing on the basis of protein charge either in the presence of ampholines (Rotofor system, BioRad) or with the use of multi-compartment electrolyzers with isoelectric immobilized pH gradient (IPG) membranes (Herbert and Righetti, 2000; Righetti et al., 2001). Preparative electrophoresis is a general method for protein purification. In previous studies, we applied preparative electrophoresis to isolate interferon γ-interferon γ receptor complexes for crystallization purposes (Fountoulakis et al., 1993; Thiel et al., 2000). We also applied preparative electrophoresis to enrich low-abundance brain proteins, possibly involved in neurological disorders (Engidawork and Lubec, 2001). The approach resulted in the enrichment of low-molecular-mass and neuron-specific proteins (Fountoulakis and Juranville,

2003). This method has also been used with acid-labile detergents for the direct mass spectrometry analysis of whole proteins (Meng et al., 2002). In this study, we used preparative electrophoresis to enrich low-abundance rat liver cytosolic proteins prior to proteomic analysis.

Materials and methods

Materials

Immobilized pH-gradient (IPG) strips were purchased from Amersham Biosciences (Uppsala, Sweden). Acrylamide was obtained from Serva (Heidelberg, Germany) and the other reagents for the polyacrylamide gel preparation were from Bio-Rad (Hercules, CA, USA). Ampholytes (Resolyte 3.5-10) were purchased from BDH Laboratory Supplies (Poole, UK). CHAPS and thiourea were from Sigma (St. Louis, MO, USA), urea, dithioerythritol and EDTA were obtained from Merck (Darmstadt, Germany).

Sample preparation

Animals were sacrificed using CO_2. Livers were flushed through the hepatic vein with cold NaCl to eliminate excessive blood content. Liver tissue (1.0 g) was suspended in 10 ml of 20 mM Hepes-OH, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 5 mM dithierythritol and 1 μl/ml of a mixture of protease inhibitors (1 mM PMSF and 1 tablet complete^{TM} (Roche Diagnostics, Mannheim, Germany) per 50 ml of suspension buffer) and phosphatase inhibitors (0.2 mM Na_2VO_3 and 1 mM NaF). The suspension was homogenized with the use of a teflon/potter homogenizer and centrifuged at $800 \times g$ for 10 min to remove nuclei and undissolved material. The supernatant was centrifuged at $10000 \times g$ for 15 min to separate the mitochondrial proteins. The supernatant of this centrifugation step was centrifuged further at $100000 \times g$ for 1 h to separate cytosolic and microsomal proteins. The cytosolic fraction was concentrated 10-fold by ultrafiltration (Mr cut off 10000), diluted 10-fold with 50 mM Tris-HCl, pH 6.8, containing 25% glycerol and 1% lithium dodecyl sulfate (LDS) and concentrated again to reach a protein concentration of about 12.5 mg/ml. The protein content was determined using the Coomassie blue method (Bradford, 1976).

Preparative electrophoresis

Preparative gel electrophoresis was performed in the PrepCell system (Bio-Rad), following the instructions of the supplier as previously described (Fountoulakis et al., 1993). The acrylamide concentration of the cylindrical separation gel was 11% and the gel was about 6 cm long. The stacking gel had an acrylamide concentration of 4% and was 2.5 cm long. 50 mg of total proteins in 4 ml of 50 mM Tris-HCl, pH 6.8, containing 25% glycerol and 1% LDS were applied onto the stacking gel. Electrophoresis was performed at 250 V in 0.198 M glycine and 25 mM Tris, containing 0.1% LDS. Fractions started to be collected after the bromphenol blue front reached the lower end of the gel. The eluted proteins were collected from the gel in 0.198 M glycine and 25 mM Tris, containing 0.1% CHAPS at 30 ml/h. Eighty 10-ml fractions were collected and each fraction was concentrated to about 0.2 ml by ultrafiltration. Excess of salt and LDS were reduced by twice diluting the concentrated sample 10-fold with 20 mM Tris-HCl, pH 7.5, containing 8 M urea and 4% CHAPS and concentrating it by ultrafiltration. Approximately 12 mg of protein was recovered in all fractions.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed essentially as reported (Langen et al., 1997). Samples from selected fractions, containing 0.1–1 mg of total protein, were applied on immobilized pH 3–10 nonlinear gradient strips in sample cups at their basic and acidic ends. Focusing started at 200 V and the voltage was gradually increased to 5000 V at 3 V/min and kept constant for a further 24 h. The second-dimensional separation was performed on 12% SDS polyacrylamide gels ($180 \times 200 \times 1.5$ mm) run at 40 mA per gel, in an ISO-DALT apparatus. After protein fixation for 12 h in 40% methanol, containing 5% phosphoric acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 24 h. Molecular masses were determined by running standard protein markers (Gibco, Basel, Switzerland), covering the range 10–220 kDa. pI values were used as given by the supplier of the IPG strips. Excess of dye was washed from the gels with H_2O and the gels were scanned in an Agfa DUOSCAN densitometer (resolution 200). Electronic images of the gels were recorded using Photoshop (Adobe) software. The images were stored as both tiff (about 5 Mbytes/file) and jpeg (about 50 Kbytes/file) formats. The figures were prepared with PowerPoint (Microsoft) software.

Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS)

MALDI-MS analysis was performed as described elsewhere (Fountoulakis and Langen, 1997) with certain modifications (Jiang et al., 2003). The spots from selected gels were excised with a spot picker and placed into 96-well microtiter plates. Each spot was destained with 100 μl of 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a speedvac evaporator. Each dried gel piece was rehydrated with 4 μl of 3 mM Tris-HCl, pH 9.0, containing 50 ng trypsin (Roche Diagnostics). After 16 h at room temperature, 7 μl of H_2O were added to each gel piece and the samples were shaken for 10 min. Four μl of 50% acetonitrile, containing 0.3% trifluoroacetic acid and the standard peptides des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da), in water were added to each gel piece. The application of the samples was performed with a Cy-Well apparatus (Cybio AG, Jena, Germany). 1.5 μl of the peptide mixture was simultaneously applied with 1 μl of matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, containing 0.1% trifluoroacetic acid. Samples were analyzed in a time-of-flight mass spectrometer (Reflex 3, Bruker Daltonics, Bremen, Germany). An accelerating voltage of 20 kV was used. Peptide matching and protein searches were performed automatically with the use of in-house developed software (Berndt et al., 1999). The peptide masses were compared to the theoretical peptide masses of all available proteins from all species. Monoisotopic masses were used and a mass tolerance of 0.0025% was allowed. The probability of a false positive match with a given MS-spectrum was determined for each analysis. Four matching peptides was the minimal requirement for an identity assignment. Unmatched peptides or miscleavage sites were not considered. The automatically identified proteins were checked individually and only rat proteins or highly homologous counterparts from other species (mouse or human) with pI and Mr values close to the theoretical were considered (a deviation of about 20% was allowed).

Results

Protein fractionation and enrichment by preparative electrophoresis

Rat liver cytosolic proteins (50 mg) were fractionated over a cylindrical 11% acrylamide gel. The sample was applied in 1% lithium dodecyl sulfate (LDS) and the elecrophoresis was performed in the presence of 0.1% LDS. The proteins as they were eluted from the gel were collected

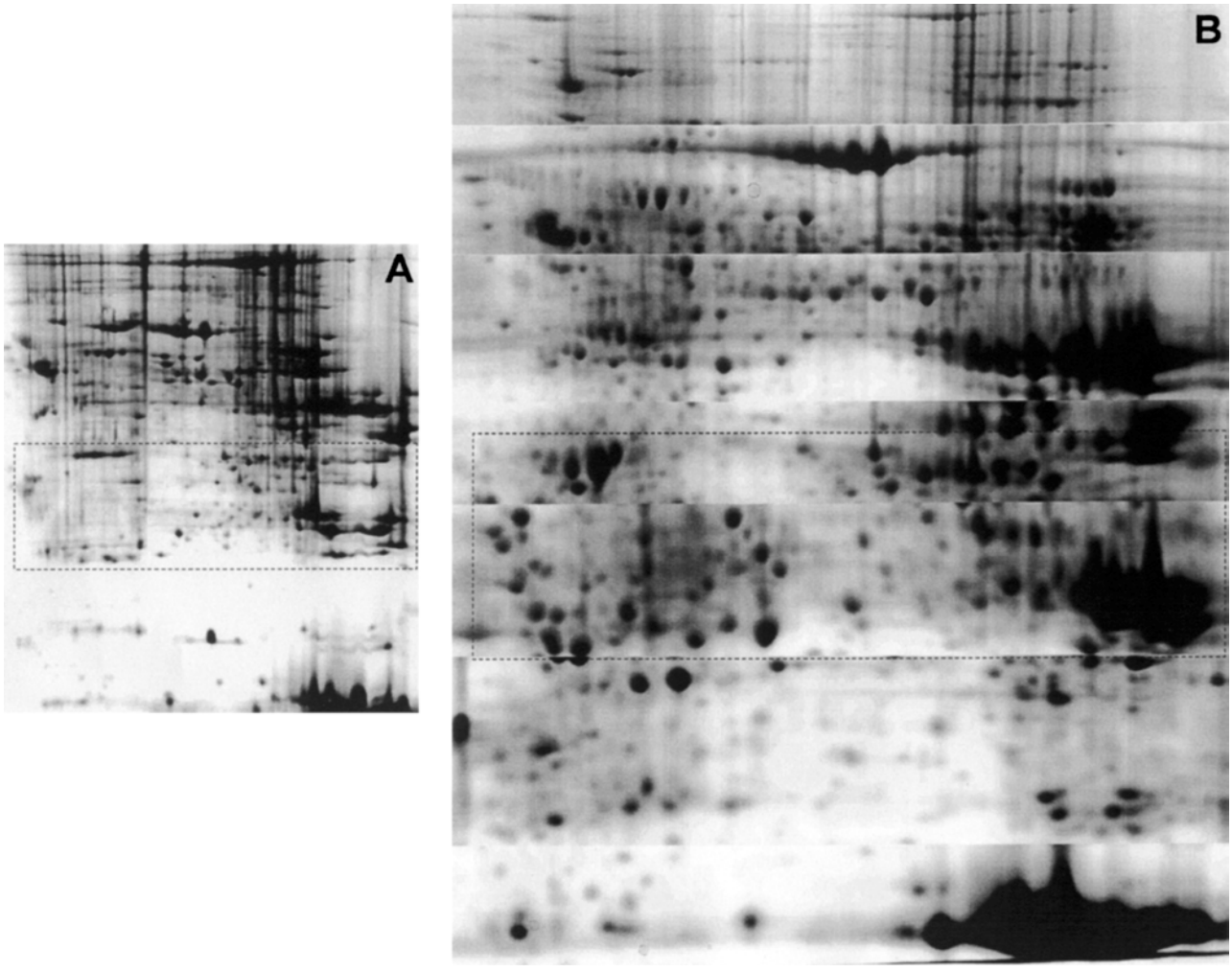

Fig. 1. Two-dimensional gel analysis of rat liver cytosolic proteins (**A**) and reconstructed 2-D gel from partial images of selected fractions, which were collected from the PrepCell (**B**). In the reconstructed gel (**B**), stronger spots representing proteins enriched by preparative electrophoresis are seen in comparison with the starting material (**A**). The rectangles show regions with significant differences in spot number and intensity. The proteins were analyzed as stated in Materials and methods. The gels were stained with colloidal Coomassie blue

in a buffer containing 0.1% CHAPS to avoid eventual precipitation. The eluate was concentrated by ultrafiltration. Simultaneously salt was removed and LDS was exchanged against CHAPS, an isoelectric focusing compatible detergent. Total protein recovery from the preparative gel (after ultrafiltration and buffer exchange) was approximately 25%. We used LDS instead of SDS because in control experiments we had observed that LDS can be easier removed from the proteins in comparison with SDS and it does not interfere with 2-D electrophoresis, whereas SDS can not be completely removed and often produces horizontal streaking in the gels (Fountoulakis and Takács, 2001).

Selected fractions were concentrated and subsequently analyzed by 2-D electrophoresis in broad pH range 3–10 nonlinear IPG strips. Figure 1A shows the 2-D gel analysis of the cytosolic fraction applied on the preparative gel and Fig. 1B shows an artificial gel of the eluate, reconstructed from the 2-D gel analysis of the fractions collected from the preparative gel. In the artificial gel, a larger number of spots can be seen in comparison with the starting material. Comparison of the spots included in the rectangles drawn in Fig. 1A and 1B shows that after the preparative electrophoresis step the spots are much stronger and new spots can be detected.

In Fig. 2, the 2-D gel analyses of the starting material and of selected fractions collected from the preparative electrophoresis step are shown. In the latter gels, in which consecutive fractions were analyzed, the spots form zones with gradually increasing average molecular masses. Preparative

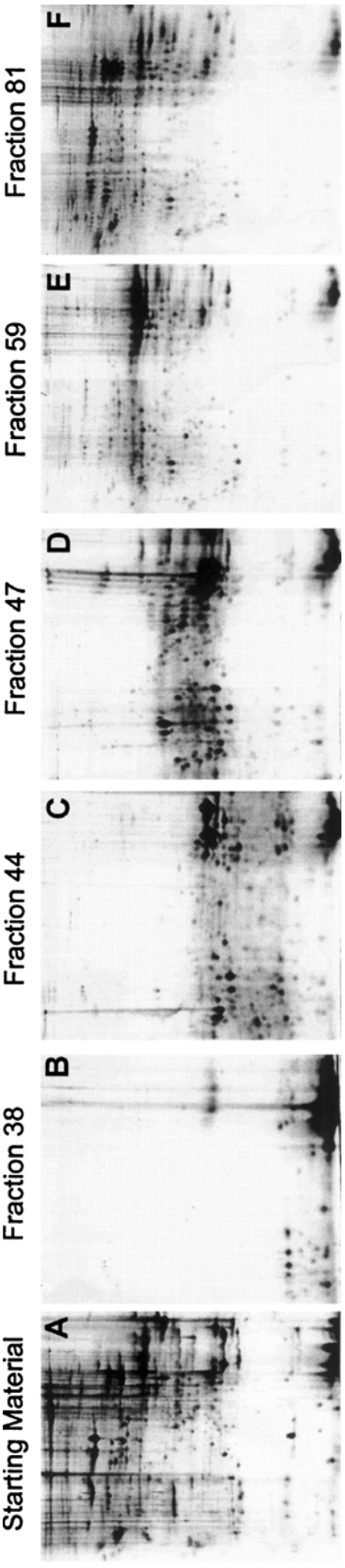

Fig. 2. Two-dimensional gel analysis of rat liver cytosolic proteins (**A**) and of the fractions 38, 44, 47, 59 and 81 eluted from the PrepCell (**B–F**). In the fractions (**B–F**), stronger spots, distributed in zones with increasing average molecular masses, are seen. The proteins were analyzed as stated under the legend to Fig. 1

electrophoresis mainly enriched proteins of average molecular mass (about 20–40 kDa, Fig. 2D). Enrichment of low-molecular-mass proteins was also efficient (Fig. 2B–C). The enrichment of high-molecular-mass proteins (Fig. 2F) was less efficient compared to those of lower molecular masses. This can be due to technical limitations of the preparative electrophoresis or very large volumes are required for their elution.

Several mainly low- and medium-size proteins, which had not been detected before, were detected in the fractions collected from the preparative gel. Five proteins were detected for the first time. These were represented by one spot. The other proteins had been found in at least one additional sample analyzed in our laboratory, ten of them in more than 100 samples. The often detected proteins were usually represented by more than one spot in the fractions from the preparative electrophoresis, 20 of them by more than 10 spots. In general, low- and high-abundance proteins were simultaneously enriched by preparative electrophoresis. Preparative electrophoresis resulted in the enrichment of glutathione S-transferase yb1 (P04905), glutathione S-transferase yb2 (P08010), carbonic anhydrase (P14141), catechol O-methyltransferase (P22734), senescence marker protein-30 (Q03336) and other proteins (Fig. 3).

The proteins collected from the preparative electrophoresis are structural molecules (about 15), heat shock proteins (about 10), enzymes involved in carbohydrate metabolism (about 15, like fructose-bisphosphate aldolase b, fructose-1,6-bisphosphatase, peroxisomal bifunctional enzyme, aldehyde dehydrogenase and others), in amino acid metabolism (about 20), involved in phenylalanine catabolism, arginine biosynthesis, glycine biosynthesis and other pathways. Proteins were also detected which are involved in response to oxidative stress (major urinary protein, thioredoxin peroxidase 1 and 2), in signal transduction (about 10, like probable protein disulfide isomerase, 14-3-3 proteins), translation and translation regulation, transport (about 20; electron, ion, oxygen transporters) and other functions.

Protein identification

The proteins were identified by MALDI-MS on the basis of peptide mass matching (Henzel et al., 1993; Lahm and Langen, 2000), following in-gel digestion with trypsin. Spots were excised from selected 2-D gels and each spot was analyzed individually. The peptide masses were matched with the theoretical peptide masses of all known proteins from all species. The analysis resulted in the identification of about 600 proteins, which were the products of 140 different genes. The number of the different gene

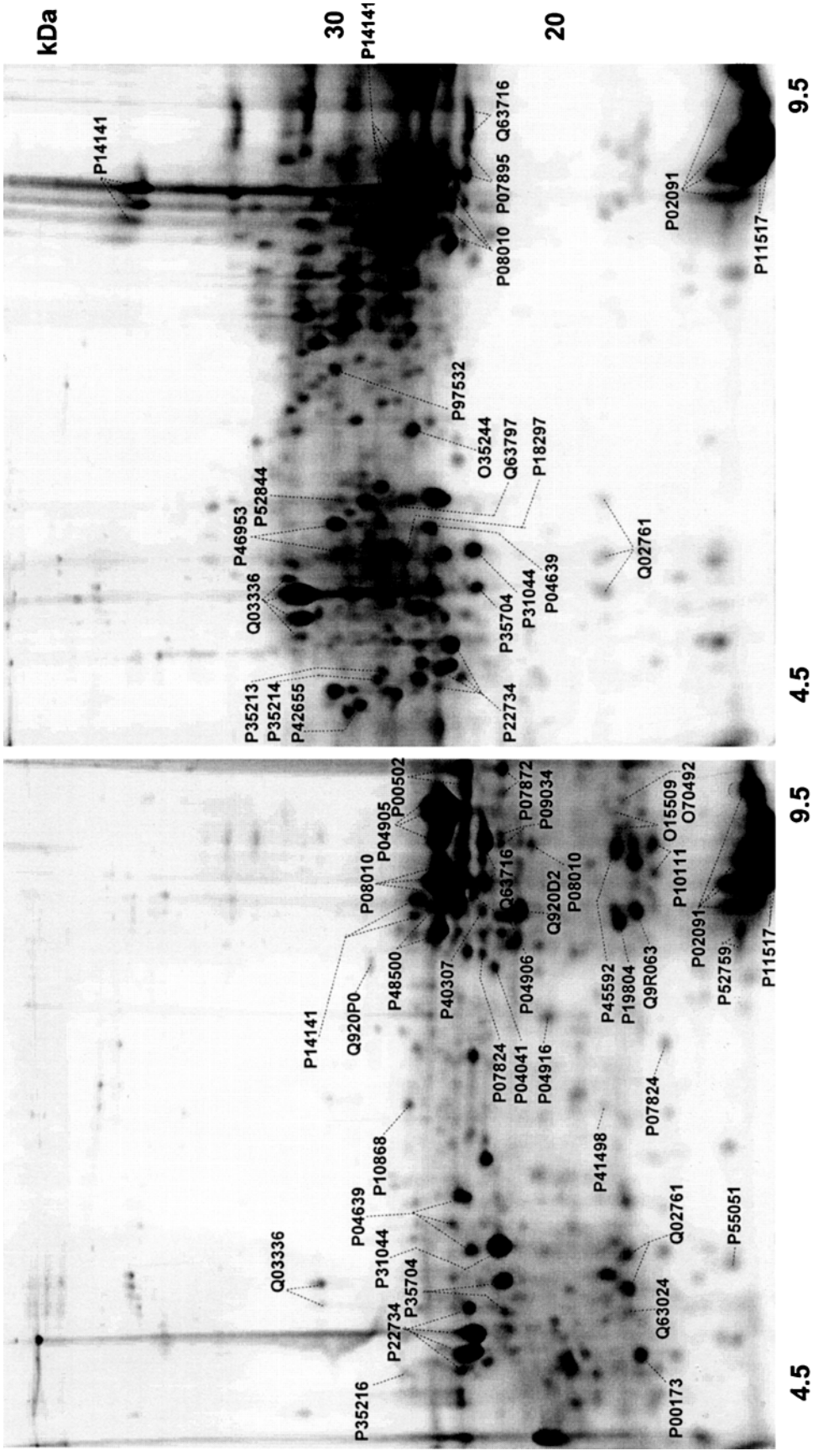

Fig. 3. Two-dimensional gel analysis of the fractions 44 and 47 eluted from the PrepCell. The gels were stained with colloidal Coomassie blue. The identities assigned are listed in Table 1

Table 1. Rat liver proteins

Number	Protein	Full name	pI	MW	Matches	Probability
O08557	SW:DDH1_RAT	Ng,Ng-dimethylarginine dimethylaminohydrolase 1 (EC 3.5.3.18) (dimethylargininase 1) (dimethylarginine dimethylaminohydrolase 1)	6.07	31805	5	2.08E-05
O09171	SW:BHMT_RAT	Betaine–homocysteine S-methyltransferase (EC 2.1.1.5)	7.89	45403	8	4.32E-11
O15509	SW:AR20_HUMAN	ARP2/3 complex 20 kDa subunit (p20-arc)	8.65	19768	4	8.22E-06
O35077	SW:GPDA_RAT	Glycerol-3-phosphate dehydrogenase (nad+), cytoplasmic (EC 1.1.1.8) (gpd-c) (gpdh-c)	6.76	37869	5	3.80E-05
O35244	SWTR_AOX2_RAT	Antioxidant protein 2 (EC 1.11.1.7) (acidic calcium-independent phospholipase a2) (thiol-specific antioxidant protein)	5.78	24728	4	2.00E-04
O35987	SWTR_RAT:O35987	P47, complete CDS	4.90	40655	6	6.81E-06
O60493	SW:SNX3_HUMAN	Sorting nexin 3 (sdp3 protein)	9.19	18807	4	7.40E-06
O88618	SW:FTCD_RAT	Formiminotransferase-cyclodeaminase (58 kDa microtubule-binding protein) [includes: glutamate formiminotransferase (EC 2.1.2.5)	5.99	59533	10	1.07E-12
O88989	SWTR_RAT:O88989	Malate dehydrogenase (EC 1.1.1.37)	6.54	36631	5	1.20E-04
P00173	SW:CYB5_RAT	Cytochrome b5	4.74	15214	5	6.05E-10
P00481	SW:OTC_RAT	Ornithine carbamoyltransferase precursor (EC 2.1.3.3) (otcase) (ornithine transcarbamylase)	9.91	39917	8	2.22E-08
P00507	SW:AATM_RAT	Aspartate aminotransferase, mitochondrial (EC 2.6.1.1) (transaminase a) (glutamate oxaloacetate transaminase-2)	9.62	47683	7	1.67E-05
P00884	SW:ALFB_RAT	Fructose-bisphosphate aldolase b (EC 4.1.2.13) (liver-type aldolase)	8.43	39918	6	4.63E-06
P00920	SW:CAH2_MOUSE	Carbonic anhydrase II (EC 4.2.1.1) (carbonate dehydratase ii)	7.05	29056	6	2.24E-06
P01946	SW:HBA_RAT	Hemoglobin alpha-1 and alpha-2 chains	8.03	15358	5	1.61E-05
P02081	SW:HBBF_BOVIN	Hemoglobin beta fetal chain (hemoglobin gamma chain)	7.05	15963	7	4.40E-08
P02091	SW:HBB1_RAT	Hemoglobin beta chain, major-form	8.19	15952	8	2.37E-10
P02551	SW:TBA1_MOUSE	Tubulin alpha-1 chain	4.81	50787	5	4.27E-07
P02650	SW:APE_RAT	Apolipoprotein E precursor (apo-e)	5.06	35788	6	6.70E-07
P02761	SW:MUP_RAT	Major urinary protein precursor (mup) (alpha-2u-globulin) (15.5 kd fatty acid binding protein) (15.5 kd fabp)	6.15	21008	6	6.75E-11
P02770	SW:ALBU_RAT	Serum albumin precursor	6.44	70669	9	9.76E-12
P04041	SW:GSHC_RAT	Glutathione peroxidase (EC 1.11.1.9) (gshpx-1) (cellular glutathione peroxidase)	7.80	22472	5	1.44E-06
P04176	SW:PH4H_RAT	Phenylalanine-4-hydroxylase (EC 1.14.16.1) (pah) (phe-4-monooxygenase)	6.03	52302	9	3.19E-09
P04639	SW:APA1_RAT	Apolipoprotein A-I precursor (apo-ai)	5.55	30126	6	1.81E-08
P04642	SW:LDHM_RAT	L-lactate dehydrogenase m chain (EC 1.1.1.27) (ldh-a)	8.34	36712	8	1.75E-09
P04691	SW:TBB1_RAT	Tubulin beta chain (t beta-15)	4.63	50387	6	5.34E-06
P04762	SW:CATA_RAT	Catalase (EC 1.11.1.6)	7.51	60061	11	5.81E-13
P04764	SW:ENOA_RAT	Alpha enolase (EC 4.2.1.11) (2-phospho-d-glycerate hydro-lyase) (non-neural enolase) (nne)	6.54	47297	7	1.06E-06
P04785	SW:PDI_RAT	R protein disulfide isomerase (PDI) (EC 5.3.4.1)/prolyl 4-hydroxylase beta (EC 1.14.11.2)/thyroid hormone binding protein/thyroxine deiodinase (EC 3.8.1.4)	4.66	57314	10	9.07E-11
P04797	SW:G3P_RAT	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) (gapdh)	8.34	35967	4	7.50E-05
P04904	SW:GTC1_RAT	Glutathione s-transferase yc-1 (EC 2.5.1.18) (chain 2) (gst yc1) (gst class-alpha)	9.47	25229	6	5.47E-06
P04905	SW:GTM1_RAT	Glutathione s-transferase yb1 (EC 2.5.1.18) (chain 3) (class-mu)	8.35	25937	9	2.18E-14
P04906	SW:GTP_RAT	Glutathione s-transferase p (EC 2.5.1.18) (gst 7–7) (chain 7) (class-pi)	7.37	23521	5	3.20E-06
P04916	SW:RETB_RAT	Plasma retinol-binding protein precursor (prbp) (rbp)	5.83	23547	4	7.56E-05
P05197	SW:EF2_RAT	Elongation factor 2 (ef-2)	6.81	96192	6	4.94E-07
P05217	SW:TBB2_HUMAN	Tubulin beta-2 chain	4.63	50255	6	1.78E-04
P05218	SW:TBB5_MOUSE	Tubulin beta-5 chain	4.62	50095	6	1.73E-04
P05544	SW:CPI3_RAT	Contrapsin-like protease inhibitor 3 (cpi-23) (serine protease inhibitor 1) (spi-1)	5.46	46419	7	1.51E-08
P05545	SW:CPI1_RAT	R contrapsin-like protease inhibitor (cpi-21) (kallikrein-binding protein) (growth hormone-regulated proteinase inhibitor) (serine protease inhibitor 2)	5.21	46760	9	4.16E-10
P05784	SW:K1CR_MOUSE	Keratin, type i cytoskeletal 18 (cytokeratin 18) (cytokeratin endo b) (keratin d)	5.08	47344	6	1.83E-04
P06757	SW:ADHA_RAT	Alcohol dehydrogenase a chain (EC 1.1.1.1)	8.08	40400	4	1.13E-05
P06761	SW:GR78_RAT	78 kDa glucose regulated protein (grp 78) (immunoglobulin heavy chain binding protein) (bip) (steroidogenesis-activator polypeptide)	4.90	72473	5	1.45E-04
P07756	SW:CPSM_RAT	Carbamoyl-phosphate synthase [ammonia] mitochondrial (EC 6.3.4.16) (carbamoyl-phosphate synthetase i)	6.75	165672	11	4.04E-09
P07824	SW:ARGI_RAT	Arginase 1 (EC 3.5.3.1) (liver-type arginase). 7/98	7.28	35122	8	3.60E-10
P07872	SW:CAOP_RAT	Acyl-coenzyme A oxidase, peroxisomal (EC 1.3.3.6) (palmitoyl-coa oxidase) (aox)	8.71	75030	7	7.29E-08
P07896	SW:ECHP_RAT	Peroxisomal bifunctonal enzyme [enoyl-CoA hydratase (EC 4.2.1.17); 3,2-trans-enoyl-CoA isomerase (EC 5.3.3.8); 3-hydroxyacyl-CoA dehydrog (EC 1.1.1.35)]	9.96	79047	6	2.63E-07
P08009	SW:GTM3_RAT	Glutathione s-transferase yb3 (EC 2.5.1.18) (chain 4) (class-mu)	7.37	25704	5	2.39E-06
P08010	SW:GTM2_RAT	Glutathione s-transferase yb2 (EC 2.5.1.18) (chain 4) (class-mu)	7.43	25725	9	1.09E-14
P08109	SW:HS7C_RAT	Heat shock cognate 71 kDa protein	5.26	71055	13	5.37E-15
P09034	SW:ASSY_RAT	Argininosuccinate synthase (EC 6.3.4.5) (citrulline-aspartate ligase)	7.83	46752	6	6.81E-09
P09244	SW:TBB7_CHICK	Tubulin beta-7 chain (tubulin beta 4′)	4.62	50095	6	1.73E-04

P10111	SW:CYPH_RAT	Peptidyl-prolyl cis-trans isomerase a (EC 5.2.1.8) (ppiase) (rotamase) (cyclophilin a) (cyclosporin a-binding protein) (p31)	8.19	17959	6	9.99E-11
P10719	SW:ATPB_RAT	ATP synthase beta chain, mitochondrial precursor (EC 3.6.1.34)	5.09	56318	9	3.73E-10
P10760	SW:SAHH_RAT	Adenosylhomocysteinase (EC 3.3.1.1) (s-adenosyl-l-homocysteine hydrolase) (adohcyase)	6.51	47889	9	7.34E-09
P10860	SW:DHE3_RAT	Glutamate dehydrogenase (EC 1.4.1.3) (gdh)	8.04	61731	10	4.11E-10
P10868	SW:GAMT_RAT	Guanidinoacetate n-methyltransferase (EC 2.1.1.2)	6.04	26544	5	2.75E-06
P11517	SW:HBB2_RAT	Hemoglobin beta chain, minor-form	9.25	15955	8	4.13E-13
P11598	SW:ER60_RAT	Probable protein disulfide isomerase ER-60 (ec 5.3.4.1) (erp60) (58 kDa microsomal protein) (p58) (hip-70) (q-2)	6.14	57043	9	1.59E-10
P11884	SW:DHAM_RAT	Aldehyde dehydrogenase, mitochondrial (EC 1.2.1.3) (class 2) (aldh1) (aldh-e2)	7.02	56965	7	7.57E-08
P12346	SW:TRFE_RAT	Serotransferrin precursor (siderophilin) (beta-1-metal binding globulin)	7.12	78538	5	1.00E-06
P12928	SW:KPYR_RAT	Pyruvate kinase, isozymes r/l (EC 2.7.1.40) (l-pk)	6.90	62503	4	1.61E-04
P13255	SW:GLMT_RAT	Glycine N-methyltransferase (EC 2.1.1.20) (folate-binding protein)	7.45	32796	7	1.53E-07
P13437	SW:THIM_RAT	3-Ketoacyl-CoA thiolase mitochondrial (EC 2.3.1.16) (beta-ketothiolase) (acetyl-CoA acyltransferase) (mitochondrial 3-oxoacyl-CoA thiolase)	7.92	42243	9	2.41E-09
P13444	SW:METL_RAT	S-adenosylmethionine synthetase alpha and beta forms (EC 2.5.1.6) (methionine adenosyltransferase) (adomet synthetase)	5.83	44240	7	1.74E-09
P13645	SW:K1CJ_HUMAN	Keratin, type i cytoskeletal 10 (cytokeratin 10) (k10) (ck 10)	4.99	59710	6	6.37E-07
P14141	SW:CAH3_RAT	Carbonic anhydrase III (EC 4.2.1.1) (carbonate dehydratase iii)	6.99	29536	8	5.58E-12
P14408	SW:FUMH_RAT	Fumarate hydratase, mitochondrial precursor (EC 4.2.1.2) (fumarase)	9.59	54714	5	1.64E-04
P14659	SW:HS72_RAT	Heat shock-related 70 kDa protein 2 (heat shock protein 70.2) (testis-specific heat shock protein-related) (hst)	5.34	69770	5	2.24E-05
P14669	SW:ANX3_RAT	Annexin iii (lipocortin iii) (placental anticoagulant protein iii) (pap-iii) (35-alpha calcimedin)	6.42	36527	5	3.39E-05
P17475	SW:A1AT_RAT	Alpha-1-antiproteinase precursor (alpha-1-antitrypsin) (alpha-1-proteinase inhibitor)	6.00	46277	5	1.50E-06
P17988	SW:SUAR_RAT	Aryl sulfotransferase (EC 2.8.2.1) (phenol sulfotransferase) (pst-1) (sulfokinase) (aryl sulfotransferase iv) (astiv) (tyrosine-ester sulfotransferase)	6.85	34169	5	1.73E-07
P18297	SW:SPRE_RAT	Sepiapterin reductase (EC 1.1.1.153) (spr)	5.46	28509	6	1.46E-06
P18421	SW:PRC5_RAT	Proteasome component C5 (EC 3.4.99.46) (macropain subunit c5) (proteasome gamma chain) (multicatalytic endopeptidase complex subunit c5)	7.38	26690	7	6.99E-09
P18757	SW:CGL_RAT	Cystathionine gamma-lyase (EC 4.4.1.1) (gamma-cystathionase) (probasin-related antigen) (prb-ra)	7.89	44261	6	6.06E-08
P19112	SW:F16P_RAT	Fructose-1,6-bisphosphatase (EC 3.1.3.11) (d-fructose-1,6-bisphosphate 1-phosphohydrolase) (fbpase)	5.56	39909	8	1.26E-08
P19226	SW:P60_MOUSE	Mitochondrial matrix protein P1 (p60 lymphocyte protein) (60 kd chaperonin) (heat shock protein 60) (hsp-60) (protein cpn60) (groel protein) (hsp-65)	6.02	61088	9	1.59E-08
P19804	SW:NDKB_RAT	Nucleoside diphosphate kinase b (EC 2.7.4.6) (ndk b) (ndp kinase b) (p18)	7.51	17385	6	1.04E-09
P20059	SW:HEMO_RAT	Hemopexin precursor	7.59	51999	8	7.45E-08
P20673	SW:ARLY_RAT	Argininosuccinate lyase (EC 4.3.2.1) (arginosuccinase) (asal)	6.38	51745	5	4.68E-05
P22734	SW:COMT_RAT	Catechol O-methyltransferase, membrane-bound form (EC 2.1.1.6) (mb-comt) (contains: catechol O-methyltransferase, soluble form (s-comt))	5.41	29806	7	2.48E-09
P22791	SW:HMCM_RAT	Hydroxymethylglutaryl-CoA synthase, mitochondrial (EC 4.1.3.5) (hmg-CoA synthase) (3-hydroxy-3-methylglutaryl coenzyme a synthase)	8.98	57331	7	1.65E-07
P23457	SW:DIDH_RAT	3-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.50) (3-alpha-hsd) (hydroxyprostaglandin dehydrogenase)	7.07	37517	6	1.21E-08
P24329	SW:THTR_RAT	Thiosulfate sulfurtransferase (EC 2.8.1.1) (rhodanese) (fragment)	7.89	33383	6	2.61E-06
P25093	SW:FAAA_RAT	Fumarylacetoacetase (EC 3.7.1.2) (fumarylacetoacetate hydrolase) (beta-diketonase) (faa)	7.16	46231	8	1.12E-08
P27867	SW:DHSO_RAT	Sorbitol dehydrogenase (EC 1.1.1.14) (l-iditol 2-dehydrogenase)	7.22	43377	6	1.81E-07
P28037	SW:FTDH_RAT	10-Formyltetrahydrofolate dehydrogenase (EC 1.5.1.6) (fbp-ci)	6.04	99976	5	5.09E-09
P29562	SW:IF41_RABIT	Eukaryotic initiation factor 4a–i (eif-4a–i) (fragment)	5.21	45018	8	5.64E-06
P30153	SW:2AAA_HUMA	Protein phosphatase pp2a, 65 kDa regulatory subunit, alpha isoform (protein phosphatase pp2a subunit a, alpha isoform) (pr65-alpha)	4.82	65849	6	8.75E-08
P30713	SW:GTT2_RAT	Glutathione S-transferase yrs–yrs (EC 2.5.1.18) (gst 12–12) (glutathione S-transferase subunit 12) (gst class-theta)	7.98	27461	6	3.34E-08
P31044	SW:PBP_RAT	Phosphatidylethanolamine-binding protein (23 kDa morphine-binding protein) (p23k)	5.63	20902	5	9.20E-06
P31210	SW:3O5B_RAT	3-Oxo-5-beta-steroid 4-dehydrogenase (EC 1.3.99.6) (delta(4)-3-ketosteroid 5-beta-reductase)	6.61	37639	9	1.16E-13
P32261	SW:ANT3_MOUSE	Antithrombin-iii precursor (atiii)	6.40	52483	5	1.43E-04
P32755	SW:HPPD_RAT	4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) (4hppd) (hpd) (f protein) (f alloantigent) (fragment)	6.76	43591	4	8.60E-05
P34058	SW:HS9B_RAT	Heat shock protein hsp 90-beta (hsp 84)	4.90	83475	7	1.14E-04
P35213	SW:143B_RAT	14-3-3 protein beta/alpha (protein kinase c inhibitor protein-1) (kcip-1) (prepronerve growth factor rnh-1)	4.64	28019	6	8.43E-06
P35214	SW:143G_RAT	14-3-3 protein gamma (protein kinase c inhibitor protein-1) (kcip-1)	4.63	28324	6	4.20E-06
P35215	SW:143Z_MOUSE	14-3-3 protein zeta/delta (protein kinase c inhibitor protein-1) (kcip-1) (mitochondrial import stimulation factor s1 subunit)	4.55	27924	4	6.03E-05
P35216	SW:143T_MOUSE	14-3-3 protein tau (14-3-3 protein theta)	4.52	28045	4	1.46E-05
P35704	SW:TDX1_RAT	Thioredoxin peroxidase 1 (thioredoxin-dependent peroxide reductase 1) (thiol-specific antioxidant protein) (tsa)	5.35	21941	5	3.29E-07
P38983	SW:RSP4_RAT	40S ribosomal protein sa (p40) (34/67 kd laminin receptor)	4.63	32917	4	4.58E-05
P40307	SW:PRCG_RAT	Proteasome component c7-i (EC 3.4.99.46) (macropain subunit c7-i) (multicatalytic endopeptidase complex subunit c7-i)	7.49	23068	4	2.47E-05
P41034	SW:TTPA_RAT	Alpha-tocopherol transfer protein (alpha-ttp)	7.03	31939	4	4.12E-05
P41498	SW:PPAC_RAT	Low molecular weight phosphotyrosine protein phosphatase acp1/acp2 (EC 3.1.3.48) (low molecular weight cytos. acid phosphatase) (EC 3.1.3.2) (ptpase)	6.48	18696	4	1.98E-07
P41562	SW:IDHC_RAT	Isocitrate dehydrogenase (nadp) cytoplasmic (EC 1.1.1.42) (oxalosuccinate decarboxylase) (idh) (nadp ± specific icdh)	6.99	47046	10	1.08E-09
P42655	SW:143E_HUMAN	14-3-3 protein epsilon (mitochondrial import stimulation factor l subunit) (protein kinase c inhibitor protein-1) (kcip-1)	4.46	29326	5	1.62E-06

(*continued*)

Table 1 (*continued*)

Number	Protein	Full name	pI	MW	Matches	Probability
P45592	SW:COF1_RAT	Cofilin, non-muscle isoform	8.16	18748	4	2.65E-05
P46413	SW:GSHB_RAT	Glutathione synthetase (EC 6.3.2.3) (glutathione synthase) (gsh synthetase) (gsh-s)	5.48	52597	5	1.56E-05
P46462	SW:TERA_RAT	Transitional endoplasmic reticulum ATPase (ter atpase) (15s mg(2+)-atpase p97 subunit) (valosin containing protein)	4.99	89976	5	4.59E-05
P46844	SW:BIEA_RAT	Biliverdin reductase a precursor (EC 1.3.1.24) (biliverdin-ix alpha-reductase)	6.06	33715	5	1.46E-05
P46953	SW:3HAO_RA	3-Hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6) (3-hao) (3-hydroxyanthranilic acid dioxygenase) (3-hydroxyanthranilate oxygenase)	5.71	32846	9	9.04E-11
P48500	SW:TPIS_RAT	Triosephosphate isomerase (EC 5.3.1.1) (tim)	6.84	27285	7	1.89E-10
P48721	SW:GR75_RAT	Mitochondrial stress-70 protein precursor (75 kDa glucose regulated protein) (grp 75) (peptide-binding protein 74) (pbp74) (mthsp70) (mortalin)	6.22	74098	6	4.59E-08
P50237	SW:SUAC_RAT	N-hydroxyarylamine sulfotransferase (EC 2.8.2.-) (hast-i)	6.53	35854	8	1.30E-09
P50431	SW:GLYC_MOUSE	Serine hydroxymethyltransferase, cytosolic (EC 2.1.2.1) (serine methylase) (glycine hydroxymethyltransferase) (shmt) (fragment)	7.13	35260	5	1.93E-04
P51635	SW:ALDX_RAT	Alcohol dehydrogenase (NADP(+)) (EC 1.1.1.2) (aldehyde reductase) (3-dg-reducing enzyme)	7.33	36579	7	5.59E-07
P51647	SW:DHAR_RAT	Aldehyde dehydrogenase, cytosolic 2 (EC 1.2.1.3) (aldh class 1) (retinal dehydrogenase) (raldh)	7.39	54882	7	4.93E-09
P52480	SW:KPY2_MOUSE	Pyruvate kinase, M2 isozyme (EC 2.7.1.40)	7.44	58289	6	3.16E-05
P52759	SW:UK14_RAT	14.5 kDa translational inhibitor protein (perchrolic acid soluble protein)	8.39	14220	7	2.39E-09
P52844	SW:SUO1_RAT	Estrogen sulfotransferase, isoform 1 (EC 2.8.2.4) (est-1) (sulfotransferase, estrogen-preferring) (estrone sulfotransferase)	6.00	35827	6	9.39E-08
P52847	SW:SUDY_RAT	Dopa/tyrosine sulfotransferase (EC 2.8.1.-)	8.21	35040	6	1.19E-06
P55051	SW:FABB_RAT	Fatty acid-binding protein, brain (b-fabp) (brain lipid-binding protein) (blbp)	5.37	15008	5	3.23E-09
P55260	SW:ANX4_RAT	Annexin IV (lipocortin iv) (36 kDa zymogen granule membrane associated protein) (zap36)	5.16	36063	5	1.05E-06
P79303	SW:UDP2_PIG	UTP–glucose-1-phosphate uridylyltransferase 2 (EC 2.7.7.9) (udp-glucose pyrophosphorylase 2) (udpgp 2) (ugpase 2)	8.02	56956	7	1.26E-05
P97532	SW:THTM_RAT	3-Mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (mst)	6.29	33073	9	5.43E-12
Q02253	SW:MMSA_RAT	Methylmalonate–semialdehyde dehydrogenase precursor (acylating) (EC 1.2.1.27) (mmsdh)	8.24	58226	10	2.95E-11
Q03248	SW:BUP_RAT	Beta-ureidopropionase (EC 3.5.1.6) (beta-alanine synthase) (n-carbamoyl-beta-alanine amidohydrolase)	6.92	44584	5	8.31E-06
Q03336	SW:SM30_RAT	Senescence marker protein-30 (smp-30) (regucalcin) (rc)	5.32	33937	7	1.11E-09
Q10758	SW:K2C8_RAT	Keratin, type ii cytoskeletal 8 (cytokeratin 8) (cytokeratin endo a)	5.83	53854	6	2.76E-08
Q63024	SWTR_RAT:Q63024	Alpha-2U-globulin (L TYPE)	5.42	21006	5	7.82E-06
Q63060	SW:GLPK_RAT	Glycerol kinase (EC 2.7.1.30) (ATP:glycerol 3-phosphotransferase) (glycerokinase) (gk) (atp-stimulated glucocorticoid-receptor translocation promoter)	5.48	58238	7	2.05E-06
Q63081	SW:ERP5_RAT	Probable protein disulfide isomerase p5 (EC 5.3.4.1) (calcium-binding protein 1) (cabp1) (fragment)	4.79	47589	5	1.89E-04
Q63150	SW:DPYS_RAT	Dihydropyrimidinase (EC 3.5.2.2) (dhpase) (hydantoinase) (dhp)	7.20	57309	6	5.16E-06
Q63569	SW:PRSA_RAT	26S protease regulatory subunit 6a (tat-binding protein 1) (tbp-1) (spermatogenic cell/sperm-associated tat-binding protein homolog sata)	4.97	49414	4	1.34E-04
Q63716	SW:TDX2_RAT	Thioredoxin peroxidase 2 (thioredoxin-dependent peroxide reductase 2) (heme-binding 23 kDa protein) (hbp23)	8.19	22323	9	8.85E-16
Q63797	SW:PSE1_RAT	Proteasome activator complex subunit 1 (proteasome activator 28-alpha subunit) (pa28alpha) (pa28a) (activator of multicatalytic protease subunit 1)	5.90	28730	5	1.53E-05
Q63798	SW:PSE2_RAT	Proteasome activator complex subunit 2 (proteasome activator 28-beta subunit) (pa28beta) (pa28b) (activator of multicatalytic protease subunit 2)	5.54	27068	4	8.55E-05
Q8VIF7	SWTR_RAT:Q8VIF7	Selenium-binding protein	6.56	53068	7	1.58E-06
Q9CPY7	SW:AMPL_MOUSE	Cytosol aminopeptidase (EC 3.4.11.1) (leucine aminopeptidase) (leucyl aminopeptidase) (proline aminopeptidase) (EC 3.4.11.5) (prolyl aminopeptidase)	7.03	52984	6	5.85E-05
Q9R063	SW:PDX5_RAT	Peroxiredoxin 5, mitochondrial (prx-v) (peroxisomal antioxidant enzyme) (plp) (thioredoxin peroxidase pmp20) (antioxidant enzyme b166) (aoeb166)	8.71	22506	5	2.12E-06
Q9WTT6	SW:GUAD_RAT	Guanine deaminase (EC 3.5.4.3) (guanase) (guanine aminase) (guanine aminohydrolase) (gah)	5.70	51553	6	6.38E-08

Cytosolic proteins from rat liver were prepared and separated by preparative electrophoresis as described under Materials and methods. The proteins were analyzed by 2-D electrophoresis and identified by MALDI-TOF-MS, following in-gel digestion with trypsin. The search in protein databases was performed with in house developed software (Berndt et al., 1999). At least 4 matching peptides were required for an identity assignment. The number of matching peptides is listed in Table 1 (matches). The spots representing selected proteins identified are indicated in Fig. 3 and are designated with their accession numbers of the SWISS-PROT database. The theoretical Mr and pI values, as well as the probability of assignment of a wrong protein identity are given. In the column "Protein", the abbreviated name of the protein and the database used for protein search are indicated

products appears to be relatively low in comparison with the proteins identified and this is because spots representing the same protein were present in several gels.

The identification was based on 4 to 13 matching peptides. Proteins of low molecular mass, which deliver few peptides (Fountoulakis et al., 1998), were usually identified with 4 matches. The average molecular mass of the proteins identified with four peptides was 30.7 kDa and those identified with nine or more matches 54 kDa. When the identification was based on seven or more matches, the probability of a wrongly assigned identity was usually lower than 10^{-7}. In Table 1, the proteins identified in selected fractions of the preparative gel are listed together with the theoretical MW and pI values and data from the mass spectrometry analysis, i.e. the numbers of matching peptides and the probability of assignment of a random identity. The spots representing proteins identified in selected fractions of the preparative electrophoresis are shown in the gels of Fig. 3 (not all protein identities are shown).

Discussion

Genomics and proteomics are high-throughput technologies, which can easily generate toxicity patterns, i.e. alterations in gene or protein levels resulting from the effect of toxic agents, an information which can lead to drug toxicity prediction (Steiner and Anderson, 2000; Fielden and Zacharewski, 2001). Early detection of toxic effects of drug candidates increases the performance of the drug design process and the safety of pharmaceuticals. An unambiguous relationship between toxicity and gene or protein pattern derangement has not been established yet. Up to now, mainly model compounds, such as acetaminophen (Qiu et al., 1998; Fountoulakis et al., 2000), thioacetamide (Dogru-Abbasoglu et al., 2001) or carbon tetrachloride (Stoyanovsky and Cederbaum, 1999; Fountoulakis et al., 2002b) are usually administered to animals and tissue samples are analyzed by employing the new approaches for the generation of toxicity databases, which will function as a guiding cue in predicting toxicity in similar cases. To facilitate the performance of toxicity studies and the investigation of animal models of human diseases, we constructed two-dimensional databases for rat and mouse liver cytosolic and mitochondrial proteins (Fountoulakis et al., 2000; 2001; 2002a; Fountoulakis and Suter, 2002).

We have applied proteomics technologies to study changes in the levels of liver proteins of rats treated with carbon tetrachloride (Fountoulakis et al., 2002b), of mice treated with acetaminophen (Fountoulakis et al., 2000), as well as changes of brain proteins of rats treated with the neurotoxin kainic acid, a cyclic analogue of glutamate (Krapfenbauer et al., 2001a). In all cases, the differential protein expression studies revealed the presence of significant derangements in the levels of a series of protein classes, following administration of the toxic agents. However, analysis of the proteins of the main subcellular fractions of a system is not sufficient for the detection of the majority of the low-abundance gene products which are involved in toxicity pathways. In previous studies, we have pointed out the necessity of applying chromatography steps to enrich low-abundance proteins of various organisms prior to a proteomic analysis (Fountoulakis et al., 1997; Fountoulakis, 2001; Krapfenbauer et al., 2001b; Fountoulakis and Takács, 2002). Here we applied a combination of subcellular fractionation of liver proteins and subsequent enrichment of the proteins of the cytosolic fraction by preparative electrophoresis.

The present study resulted in the identification of 140 proteins from the analysis of selected fractions collected from the preparative gel. Low- and middle-molecular-weight proteins were preferentially enriched with this method. Proteins with a theoretical molecular mass below 25 kDa represent approximately 21% of the proteins listed in Table 1. In comparison, the corresponding proteins identified from total liver extract represent about 18% of all identified proteins (Fountoulakis and Suter, 2002). The method was efficient in the enrichment of low-abundance liver proteins and of certain high-abundance protein classes, like glutathione S-transferases and 14-3-3 proteins.

Preparative electrophoresis appears to be very efficient for fractionation of cytosolic proteins. Fractionation of the microsomal proteins, after solubilization with 1% LDS, on the preparative gel was less satisfactory in comparison with the cytosolic fraction (data not shown). The membrane proteins are most likely forming strong complexes, which can not be disrupted by the resolving power of the preparative electrophoresis. In addition, hydrophobic proteins may have been missed during the 2-D gel electrophoretic analysis, as they do not enter IPG strips (Fountoulakis and Gasser, 2003). Other limitations of the approach are the relatively low recovery of total proteins eluted from the preparative gel (about 25%) and the inefficient enrichment of large proteins. Furthermore, a general limitation of the 2-D gel analysis of chromatography fractions is that proteins strongly enriched in certain fractions are represented by multiple, strong and often overlapping spots, which suppress the signals of co-eluted, low-abundance proteins and consequently the detection of such proteins may not be possible during the analysis. With exception of the high molecular mass proteins, which were not efficiently enriched, all spots present in the 2-D

gels from the starting material, were also observed after performing the preparative electrophoresis step.

In summary, we applied preparative electrophoresis to enrich low-abundance liver cytosolic proteins prior to proteomic analysis. The approach resulted in the enrichment of low-abundance proteins and of glutathione transferases and 14-3-3 proteins. Mainly small- and medium-size proteins were enriched. Preparative electrophoresis offers unique advantages over other fractionation methods and can be included in an efficient protein-enriching scheme.

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