Proteomic analysis of mouse liver plasma membrane: use of differential extraction to enrich hydrophobic membrane proteins

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Abstract

To comprehensively identify proteins of the liver plasma membrane (PM), we prepared plasma membrane from mouse liver by sucrose density grade centrifugation. An optimized extraction method for whole PM proteins and several methods of differential extraction expected to enrich hydrophobic membrane proteins were tested. The extracted PM proteins were separated by 2-dimensional electrophoresis (2-DE), and were identified by Matrix Assisted Laser Desorption/Ionization-time of Flight (MALDI-TOF) mass spectrometry , and Electrospray Ionization Quadrupole-time of Flight (ESI-Q-TOF) mass spectrometry. As the complementary method, 1DE-MS/MS was also used to identify the PM proteins. The results indicated that the optimized lysis buffer containing urea, thiourea, CHAPS and NP-40 can extract more PM proteins and that the treatments of PM samples by chloroform/methanol and sodium carbonate can enrich more hydrophobic PM proteins. In the mouse liver PM fraction, 175 non-redundant gene products were identified, of which 88 (about 50%) were integral membrane proteins with one to seven transmembrane domains. The remaining are probably membrane-associated and cytosolic proteins. The function distribution of the all identified liver PM proteins was analyzed and the enzymes, receptors and the proteins with unknown function were 40%, 12% and 9%, respectively.

1 Introduction

The liver is one of the most important organs in the body, probably second only to the brain in organ complexity; displays the main digestive function for the metabolism of most substances and undertakes a myriad of other functions such as production of red blood cells during embryonic development, production of various plasma proteins, and detoxification. Liver disease afflicts more than 10% of the world population. Liver pathogenesis remains however largely undefined and most liver diseases remain poorly diagnosed, staged and

treated. The Human Proteome Organization (HUPO) initiated Human Liver Proteome Projects in 2003 [1]. The plasma membrane (PM) regulates the exchanges of information, material and energy between cell and its environment. Numerous biochemical mechanisms are modifies at PM level upon changes of environmental or internal factors. The membrane proteins on the PM as the “doorbells” and “doorways” play the crucial roles for the functions of cells.

With the rapid expansion of protein sequence databases, mass spectrometry (MS)-based identification of proteins, which relies on the sequence database, is becoming more realistic and more reliable [2]. This emerging technique has been used to analyze proteins in whole cells and tissues of liver, and thousands of proteins expressed have been detected and identified [3-5]. However, although the recent complete sequencing of several genomes estimates that transmembrane proteins (proteins anchored into the membrane being expected) represent around 30% of total proteins [6,7], membrane proteins reported so far are much lower. Recently, many developments [8-10] were achieved in membrane proteomics. Most refinements were directed at sample preparation and involved improved solubility of membrane fractions with either organic solvents [8, 9] or nonionic/zwitterionic [10] detergents on 2-D immobilized pH gradient (IPG) gels. Multidimensional liquid chromatography [11] and one-dimensional gel coupled with mass spectrometry [12] have also been used in the study of membrane proteome alternatively, but two-dimensional gel electrophoresis (2-DE) is still the core technology due to its unrivalled power of simultaneously separation of thousands of proteins and the high-sensitivity visualization. However, solubility and low copy issues of membrane proteins remain to be the main challenge in 2DE [13]. So the development of new methodologies for membrane protein solubilization and enrichment in 2DE has become increasingly necessary.

To date, the subcellular fractions in liver have been studied, such as mitochondria [14, 15], nuclear membrane [11] and peroxisome [12], for subcellular fractions analysis reduces the

complexity of the samples to be analyzed and yields more information on the minor protein components and hydrophobic proteins. However, there is no comprehensive study concerning liver plasma membrane proteome. To date, only a few PM proteome have been studied, for example, yeast *Saccharomyces cerevisiae* [16], *Arabidopsis thaliana* [17,18] and other species [19-22], most of which were studied through 1DE-MS/MS. As is mentioned above, 2DE is still the most powerful separation method for proteins even for membrane proteins. So it is significant to develop an improved 2DE protocol for the PM proteome investigation. In the present study, we combined subcellular fractionation, integral membrane proteins enrichment, differential extraction of membrane proteins, mainly with 2DE-MS based proteomic analyses to study mouse liver PM proteome. By using the optimized lysis buffer and different extraction procedures, we developed a protocol to make more membrane proteins presented in 2DE gel and to identify more integral membrane proteins. Further more, as a complementary protocol of 2DE-MS in the study of highly hydrophobic and low abundance membrane proteins, we also studied plasma membrane proteins with 1DE-MS/MS. So this work presents, to the best of our knowledge, the first systemic investigation of mouse liver plasma membrane proteome. Our purpose is (i) to develop the methodologies for membrane protein solubilization; and (ii) to comprehensively study liver PM proteome, and identify novel mouse liver PM proteins.

2 Materials and methods

2.1 Chemicals

Immobilized pH gradient (IPG) DryStrips (3-10 linear), IPG buffer (3-10 linear), Cover fluid, Agarose and Silver Staining Kit, colloidal Coomassie blue were purchased from Amersham Pharmacia-Biotech (Uppsaia, Sweden). HEPES, Sucrose, antipain, leupeptin, DTT, Iodoacetamide, Trypsin (Proteomics Sequencing Grade), CHAPS, NP-40, ASB-14, SB3-10, CHCA and TFA were obtained from Sigma (St. Louis, MO). Acrylamide, Bis-acrylamide, Urea, Thiourea, Glycine, Tris, and SDS were from Amresco (Solon, OH,

USA). ACN is domestic product (chromatogram-grade). Other chemicals are domestic products (analytical-grade). All mice are Kunming albino mice from Hunan Medical University.

2.2 Preparation of Mouse Liver Plasma Membranes

Plasma membranes were purified according to the procedure described by Ray, T. K. [23] with some modifications. Briefly, mice were killed after starved for 18-24 hours. After removed the gallbladder and blood vessels, the liver pieces were homogenized in 8 times of the liver weight (ml/g) of cold solution A containing 50 mM HEPES, 1mM $CaCl_2$ and protease inhibitors (Final concentrations are:1mM PMSF, 2μg/mL *a*ntipain and 2μg/mL of aprotinin) with a Tissue Tearor (Biospec products, CE 2000, Mexico.) at 15000 rpm until completely liquefied. The homogenate was filtered to 50-mL conical tubes, centrifuged for 15min at 1500g *(*JA-14 rotor, Beckman, U.S.A *)* at 4℃. The supernatant was carefully poured off, and the pellet was transferred to SW-28 tubes, then mixed with 50% sucrose, adding 42% or 50% sucrose to the mixture until the concentration of sucrose to 44%, 42.3% sucrose was loaded gently to the top of 44% sucrose. Centrifuged for 2.5h at 100,000g at 4℃(SB-28 rotor, Beckman, U.S.A*)*, the plasma fraction membrane at the top of SW-28 tubes was collected and washed with solution B containing 50 mM HEPES, protease inhibitors (Final concentrations are:1mM PMSF, 2μg/mL antipain and 2μg/mL of aprotinin) for three times. Then a specimen of the sample was analyzed by Electron microscopy, the others were stored at -70 ℃ in storage buffer (solution B) .

2.3 Electron microscope analysis of the PM

A specimen of plasma membrane was fixed with 2.5% glutaraldehyde overnight at room temperature ， washed with phosphate-buffered saline，and dehydrated with alcohol (25%, 50%, 75%, 95%, and 100% in turn). For scanning transmission electron microscope, portions of sample were washed with acetate isopentester，dried at critical point，stained with gold,

and examined with an S-570 Hitachi Scanning Electron Microscope (Tokyo, Japan). The rest were washed with 50% acetone, dehydrated with 100% acetone for twice, and processed into epoxy resin. Thin sections (70nm) were collected on copper grids, stained with uranyl acetate and lead citrate and examined with an H600 Hitachi transmission electron microscope (Tokyo, Japan).

2.4 2DE-MS

2.4.1 Plasma membrane protein treatment and differential extraction

Three different lysis buffers were tested for their ability to solubilize membrane proteins, and three different procedures were tested for their ability to extract hydrophobic proteins from PM. The lysis buffer 1 contained: 8M urea, 2M Thiourea, 65mM DTT, 0.5% Pharmalytes pH 3-10, 0.5mM PMSF, 4 % w/v CHAPS, 1%NP-40. Lysis buffer 2: 7M urea, 2M Thiourea, 65mM DTT, 0.5% Pharmalytes pH 3-10, 1mM PMSF, 1%(W/V) ASB14, 40mMTris-base. Lysis buffer 3: 5M urea, 2M Thiourea, 65mM DTT, 0.5% Pharmalytes pH 3-10, 0.5mM PMSF, 2% CHAPS, 2%SB3-10.

2.4.1.1 Triton X-114 treatment

PM stored in storage buffer was centrifuged at 15000g ,4℃, 5min (centrifuge 5804 R , eppendorf, the same centrifuge was used in all subsequent experiments except special account) , and the pellet was suspended at 4mg/ml in a solution containing 2% Triton X-114 , 150mM NaCl, 10mM Tris-HCl (pH7.6) and was incubated for 15 min in ice[24]. The treated PM was centrifuged for 10 min at 10,000×g at 4℃, and the supernatant was collected and incubated at 37℃. The cloudy solution obtained was centrifuged at 1000 ×g for 10 min. The lower and upper phases were collected, precipitated with cold acetone, and resuspended in the lysis buffer 1.

2.4.1.2 Carbonate treatment

The treatment was according to [24, 10]. PM stored in storage buffer was diluted 200 times

with 0.1M Na_2CO_3 and kept on ice for 30 min. After centrifugation (15000×g for 60 min), the pellet was solubilized in three different lysis buffers respectively.

2.4.1.3 Chloroform/methanol treatment

The treatment was performed as described previously [24, 25] with slight modifications. The PM stored in storage buffer was diluted in the ratio of 1:9 of cold chloroform/methanol solutions at the ratio of 5:4(v/v). The mixture was kept on ice for 15 min before centrifugation for 20 min at 12 000×g at 4℃. The organize phase and pellet were blew by nitrogen， cooled and dried in SpeedVas, then they were extracted with lysis buffer 1.

2.4.2 2-Dimensional gel electrophoresis

2-DE was performed essentially as described in [26] with little change. The first dimension of IPG-DALT 2-DE was run on an IPG-phor isoelectric focusing system (Amersham Pharmacia Biotech). Plasma membrane proteins were mixed with a rehydration solution containing 8M urea, 2M Thiourea, 4% CHAPS, 20mM Tris-base, 0.5%(V/V) IPG buffer, pH 3-10L, 18 mM DTT and a trace of bromophenol blue, to total volume of 350μL, and applied to IPG dry strips (pH 3-10 L (180×30×0.5mm)). After rehydration for 12 hours, IEF was conduced automatically to a total of 44.1KVh at 20℃. After equilibrated, the second-dimensional run was carried out on incontinuity SDS-polyacrylamide vertical slab gels with 1 mm thick, 10-15% separation and 4.8% stacking gels in a Bio-Rad Protein II electrophoresis apparatus. After 2-DE, the gels were stained with silver nitrate, or Coomassie blue dye.

2.4.3 Image acquisition and analysis

The stained gels were scanned using Qinghua Ultraviolet scanner at an optical resolution of 300 dpi in transmission model. Spot detection, quantification and matching were performed using PDQUEST software Version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.4.4 In situ digestion of proteins

The Coomassie blue-stained protein spots were excised from preparative gels using a punch and placed into 500 μL Eppendorf tubes. The proteins were digested in-gel with trypsin as described by Hellmann *et al.* [27]. Briefly, each spot was destained with 50 μL of 50% acetonitrile（ACN） in 25mM NH_4HCO_3, incubated at 37℃ for 0.5hr and repeated one time. Then the dried gels were reduced and alkylated. The gel pieces were digested with trypsin (0.02g/L) in 25mM NH_4HCO_3 containing 10% ACN at 37℃ overnight. The digests were desalted with ZipTip™ (Millipore, Bedford, MA, USA) according to the manufacture's instructions and subjected to analysis using MALDI-TOF-MS, or ESI-Q-TOF.

2.4.5 Mass Spectrometry and protein identification

2.4.5.1 MALDI-TOF analysis

The tryptic peptide mixtures were eluted in 1μL of a solution containing 5mg/mL of alpha–cyano-4-hydroxy-cinnamic acid (CHCA), 50%acetonitrile and 0.1% TFA. Then the solution was loaded on to a 96-well target plate. The samples were air-dried. Molecular weight information of peptides was obtained by using a MALDI-TOF-MS Voyager DE-STR from Applied BioSystems (Framingham, MA, USA), equipped with nitrogen laser and operated in reflector/delay extraction mode and auto acquisition control. Only the PMF with signal intensity more than 4500 were remained. All MALDI-MS spectra were internally calibrated using trypsin autolysis fragments ($[M+H^+]$ irons of 842.51 and 2210). The peptide masses were input in software MS-FIT. The database searches were performed using the following values: MUS MUSCULE species, protein molecular weight range (1000-100000) and pI range (3-10), trypsin digest (one missed cleavage allowed), cysteines modified by carbamidomethylation, mass tolerance ±50ppm using internally calibration. The identified were based on four matching peptides and more than 14% coverage. The first hit protein was considered positive when its MOWSE scores over 1000, and the difference in MOUSE score with the second ranked one more than two orders of magnitude. Tryptic autolytic fragments

and contamination were removed from the set of data used for database search.

2.4.5.2 ESI-Q-TOF analysis

Mass spectra were obtained on a quadrupole TOF (Q-TOF) mass spectrometry (Micromass, Manchester, UK) fitted with a nanoelectrospray ionization source (Micromass). For on-line liquid chromatography mass spectrometry (LC/MS) analysis, a Waters CapLc solvent delivery system (Waters) was coupled to the mass spectrometry according to [28]. The tandem mass spectrometry (MS/MS) data was acquired by the software MassLynx (Micromass, Manchester, U.K.) and was converted to PKL files, which consists of the mass values of the precursor ions and the intensity and the mass values of the fragment ions, by the ProteinLynx (Micromass). This PKL files were analyzed using MASCOT software (www.matrixscience.com). Search parameters were set as following: Database: Swiss-Prot or NCBInr; taxonomy: MUS MUSCULUS; enzyme: trypsin, allowing up to 1 missed cleavage. Peptide mass tolerance was 1.2 Da and MS/MS mass tolerance was 0.6 Da; fixed modification parameter was carbamidomethylation, and the variable modification parameters were oxidation (Met), phosphorylation (Ser, Thr, Tyr). We basically selected the candidate peptides with probability-based Mowse scores (total score) that exceeded its threshold, indicating a significant (or extensive) homology ($p<0.05$), and referred to them as “hits”. The criteria were based on the manufacturer’s definitions (Matrix Science, Ltd.)[29]. Proteins that were identified with at least two peptides showing both a score higher than 40, were validated without any manual validation. Those with at least two peptides showing one’s score higher than 40, and the others’ higher than 20 and lower than 40 were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion. For proteins identified by only one peptide, its score must exceed 50, and its peptide sequence was systematically checked manually. When there were several hits, the first hit was selected.

2.5 One-dimensional SDS-PAGE -Mass Spectrometry

Samples of liver plasma membrane were diluted with denaturing sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS,10% mercaptoethanol, and a trace of bromophenol blue), and heated at 100℃ for 5 min. Samples were subjected to SDS-PAGE using 11.5% separation and 4.8% stacking gel. After electrophoresis, the gels were stained with Coomassie Brilliant. The protein bands were excised by hand, in-gel digested, and identified by LC-ESI-Q-TOF as described in part of 2.4.6.2. With only one difference, all the proteins

listed over the criteria were selected. The database used for searches was NCBInr

2.6 Bioinformatics annotation tools.

2.6.1 Hydropathy Calculations

All identified proteins were analyzed using SOSUI database (available at http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html)[30], which allows the calculation of the grand average of hydropathicity (GRAVY) value for a given protein. The proteins exhibiting positive GRAVY values were recognized as hydrophobic.

2.6.2 Transmembrane Mapping.

All identified proteins were examined through SOSUI [30] (available at http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). The analysis was performed to report the value of TMHMM.

2.6.3 Subcellular location of proteins

All identified proteins were researched by www.expasy.org/ Swiss-Prot and TrEMBL or through the papers of www.ncbi.nlm.nih.gov. They were also predicted through PSORT Prediction of animal [31] (available at Http://psort.nibb.ac.jp/form.html).

3 Results

3.1 Optimization of PM protein extraction

The quality of the purified plasma membrane was evaluated by electron micrographs (Fig.1). Cell coat can be distinguished from Fig.1.A. From Fig.1.B, plasma membranes are visible as long interconnected black lines with membrane attached to their ends and sides. Very short pieces of membrane, which appear as black dots, remain visible.

Extraction of proteins is perhaps the most critical step in the PM proteome analysis. The lysis buffer should be tailored to suit the hydrophobic membrane proteins. Three different lysis buffers and three different procedures of the sample treatment were tested to enrich hydrophobic membrane proteins. We evaluated the efficiencies of different treatments to enrich integral proteins by 2DE. Fig. 2(A) shows the un-treated whole plasma membrane proteins

solubilized with lysis buffer 1. Fig.2 (B) (C) (D) are the lysis buffer 1 solubilized PM proteins after the treatments of PM samples by 0.1M Na_2CO_3, $CHCl_3/CH_3OH$ (5:4v/v) and Triton X-114, respectively. Through the comparision of the spots number on the 2-DE maps, the treatments by sodium carbonate and chloroform/methanol can better enrich hydrophobic proteins (280 spots from sodium carbonate treatment, 232 spots from chloroform/methanol treatment, and Only 36 spots from triton X-114). According to the molecular weight and pI values of the increased spots, comparing with the un-treated whole membrane proteins, chloroform/methanol is better than sodium carbonate to enrich some high molecular weight, low pI and high pI proteins.

We compared the extraction power of three kinds of lysis buffer according to the total number of spots and the distributing of protein spots on the gels. The plasma membrane treated with sodium carbonate was extracted by Lysis buffer 1, 2, and 3, separated by 2DE, and stained with silver nitrate (0.1mg proteins was loaded)(Fig.2B, E, and F). Then the gels were analyzed by PDQUEST software, average of 280, 271, 251 protein spots were detected in the gels respectively. Furthermore, the efficiencies of the three lysis buffers were also compared through the Coomassie blue stained 2-DE maps of the whole PM proteins solubilized by Lysis buffer1, 2, and 3, respectively (Fig.3, 4,5). The maximum protein spots number (340 spots) was detected on the gel of the sample extracted by lysis buffer 1(Fig.3). Less numbers of spots, 300 and 290 were detected on the gels of lysis buffer 2 (Fig.4) and lysis buffer 3 (Fig.5), respectively. The resulting gels stained with Coomassie blue were used for MS analysis. More hydrophobic proteins were identified in gels extracted with lysis buffer 1. The silver- and Coomassie blue- stained gels indicated that 4%CHAPS plus 1%NP-40 is better in solubilization of high molecule weight proteins, ASB-14 is better as to low molecule weight proteins. In all, lysis buffer 1 is better than lysis buffer 2, and 3 in PM proteins extraction.

3. 2 Protein identification

3. 2.1 MS analysis

In order to identify membrane proteins as many as possible, four batches of protein spots on different gels were selected and applied to MS analysis: i) total membrane proteins solubized by lysis buffer 1 (Fig.3); ii) the proteins with low molecular weight of the sample solubilized by lysis buffer 2 (Fig.4); iii) the proteins with high pI of the sample solubilized by lysis buffer 3 (Fig.5); iv) the proteins of the sodium carbonate treating sample solubilized by lysis buffer 1 (Fig.6). To improve the precision of identification of the proteins, the gels with high load (0.5 mg proteins) were stained by Coomassie blue. Protein identification was carried out using a combination of peptide mass fingerprinting (PMF), ESI-Q-TOF-MS/MS. 226 protein spots were successfully identified and assigned to 175 genes. Many proteins were identified by MALDI-TOF-MS and ESI-Q-TOF-MS/MS simultaneously, and the same identification results were obtained (such as spot 2-7, 244, 3-33), which were marked with italic words in Table S1. A representative peptide mass-fingerprinting map of spot 1N is showed in Fig.7. Sequences of annexin V (spot 210) obtained by ESI-Q-TOF are showed in Fig.8. In order to complement 2DE and identified more integral membrane proteins, we separated whole plasma membrane by 1DE. 23 protein bands (Fig. 11) were excised and subjected to in-gel digest and analyzed by ESI-Q-TOF. 190 proteins were identified (database not list), of which, 44 proteins were transmembrane proteins with one or more transmembrane domain (Table S1 and Table1).

3. 2.2 Analysis of the identified proteins

The identified proteins were functionally categorized based on Swiss-Prot and TrEMBL annotation terms (http://us.expasy.org/sprot). About 40% of the listed proteins of the mouse liver plasma membrane is enzyme subunits with a broad spectrum of catalytic activities, such as Receptor-like Protein-tyrosine Phosphatase, RPTP (spot 14); Apoptotic protease activating factor 1 (spot 24); lipoxygenase (spot 65). The list also includes nine other classes of plasma membrane proteins, like Membrane Skeletal Proteins (9%), Ligands/Receptors (12%), Antigens/Antibodies (10%), Transporters (4%), Channel proteins (2%), Binding proteins

(7%), Chaperone (4%), Others (4%), and unknown proteins (9%) (Fig.9, Table S1).

3. 2.3 Hydrophobicity and transmembrane Mapping

Table1 and Table S1 list the grand average hydrophobicity (GRAVY) values of the identified proteins, as well as the theoretical transmembrane regions. The histogram of GRAVY values of the proteins identified by 2DE is shown in Fig. 10. Positive GRAVY scores indicate hydrophobic and negative scores indicate hydrophilic proteins. The GRAVY values usually vary in the range ±1.2. Most of proteins are hydrophilic and only about 8%proteins have positive GRAVY scores.

Approximately 28% of the proteins identified by 2DE-MS carry transmembrane domains, 27 proteins include one theoretical transmembrane region, 13 proteins carry two and 9 proteins carry three or more predicted transmembrane domains (Table 1). Myelod upregulated protein (spot 3-52) is the protein with most transmenbrane domain. The 7 or 8 -transmembrane-domain proteins are seldom detected. The two-transmembrane-domain proteins are really frequently detected proteins [32] in 2DE.

Approximately 26% of the proteins with one or more transmembrane domain were found in gels of the sample by using Lysis buffer 1, 26% and 25% from the gels by Lysis buffer 2 and 3, respectively, and 27% after treated with 0.1M Na_2CO_3 (according to the all protein spots identified (226)). After treated with Na_2CO_3, the proteins with GRAVY values above 0 are 3 (account for 17%, a little more than the total PM proteins).

3. 2.4 Subcellular location

The proteins found in mouse liver plasma membrane fraction (Table S1) should be theoretically plasma membrane proteins or plasma membrane-associated proteins. The proteins identified in this work could be divided into three main groups according to their subcellular locations. The first group comprises annotated or predicted plasma membrane proteins accounting for 51% (40 and 31 proteins by annotated in the public database and through predicted in 2DE technology routes and 39 in 1DE (Http://psort.nibb.ac.jp/form.html)

respectively). For example, Integrin beta3 subunit (spot 3-46), Keratinocyte growth factor receptor (spot 6) are type I membrane protein; Cyclophilin (spot 2-7), Myeloid upregulated protein are integral membrane protein. The second group is the proteins, which are cytoplasmic, or secreted proteins according to SWISS-PROT database or prediction (Http://psort.nibb.ac.jp/form.html). There are 52 proteins, accounting for 29% (23 proteins were annotated as cytoplasmic or secreted proteins in SWISS-PROT database). 7 proteins known to be located in other subcellular fractions such as Microbody, Endoplasmic reticulum (ER), mitochondrial membrane, nuclear membrane were also identified (Table S1).

4 Discussions

The goals of this study were (i) to compare the efficiencies of extractions of different lysis buffers and different procedures for PM protein treatments to extend the solubilization of membrane proteins, (ii) to find out what proteins are included in the plasma membrane of mouse liver, and (iii) to find the limitation of the common experimental procedures in detecting membrane proteins and to find clues of improvement for later membrane proteomic study. Our results have provided some answers to these questions.

The membrane fraction used in this study was prepared by sucrose density grades centrifugation and pellet washed steps, and included the peripheral membrane proteins and integral membrane proteins. In order to determine the optimal enrichment conditions to recover hydrophobic proteins, three different treatments were tested. Our test validated the result of Veroique Santoni [24]. The PMs treated or not treated with 0.1M Na_2CO_3 were used to compare the efficiency of lysis buffer to solubilize membrane proteins. We found that lysis buffer 1 was best among the three lysis buffers. Although when SB3-10 was combined with urea, thiourea, and CHAPS, good separations were obtained for some *E. coli* outer membrane proteins [33], linear sulfobetaines SB 3-10 due to low urea compatibility (5 M for SB 3-10), has lower power of separation of membrane proteins than ASB14 and CHAPS combined with NP-40. ASB14 containing a 14C linear alkyl tail is one of the most promising detergents,

and has been successfully used to solubilize integral membrane proteins of *E. coli* and *Arabidopsis* [34-35]. Robert Henningsen *et al.* [10] have found that myristic amidosulfobetaine (ASB-14) was better than CHAPS at solubilizing a G-protein coupled receptor. We found that when 4%CHAPS is combined with 1%NP-40, it is better than ASB-14 for extracting either total membrane or integral membrane proteins. Furthermore, CHAPS and NP-40 are much cheaper than ASB14. This can be explained by the fact that membrane proteins are so complex that any single kind of detergents could not solubilize them adequately, and Nonionic detergent (NP-40) can complement zwitterionic detergent (CHAPS) for better extraction of membrane proteins.

It was recently found by Perdersen S.K. *et al* [36] that 90% of the predicted yeast membrane proteins have the GRAVY value below 0.4 and falls into the solubility range commonly detected on 2D-gels.They concluded that the low representation of integral membrane proteins on 2-D maps is not solely a function of solubility but more probably a function of abundance. This results support our efforts to enrich hydrophobic membrane proteins through differential extraction. It is difficult to present most of the hydrophobic membrane proteins in just single 2-D map or through single extraction step. Our results showed that differential extraction by multiple systems can not only found more membrane proteins with high hydrophobicity but also can enrich more hydrophobic proteins with low abundance.

We identified unvaguely 175 gene products in the PM protein sample of the mouse liver with 2DE-MS. This number is much lower than the expected number of the total PM proteome. One main reason is that there are big differences in the relative abundance of PM proteins and the 2DE gels we used were Coomassie Blue-stained and many proteins with low abundance could not be detected. The other reason is that some very hydrophobic proteins are not solubilized in isoelectric focusing buffer or are prone to precipitation at their isoelectric point. In order to identify more hydrophobic PM proteins, we used 1DE-MS/MS strategy to be the supplementary approach in the plasma membrane protein study.

Approximately 28% of the 175 gene products detected by 2DE-MS in the membrane fraction were integral membrane proteins, carrying at least one transmembrane domain according to the database. The GRAVY values provide an indication of the hydrophobicity of the proteins, most of the proteins identified in our study, even those predicted to carry transmembrane domain(s), are hydrophilic (Table S1). Our results showed that only 8% of the proteins detected in the membrane fraction has positive GRAVY values, even after treated with 0.1M Na_2CO_3, only 17% of the proteins has positive GRAVY values. Fountoulakis M. *et al* [32] reported similar result in their work about the proteomic analysis of the PM of E. *coli*. There are two possible reasons: i) There is a relatively large portion of membrane proteins that are in fact hydrophilic; and ii) Some strong hydrophobic proteins would be lost during 2DE.

At this point we turned to 1DE separation. This method enriched our database and made the number of identified integral membrane proteins up to 88. Compared with 2DE, we identified more proteins with two or more-transmembrane domains (up to 66% in 1DE, compared with 45% in 2DE, Table 1).

We found several discrepancies between annotated and predicted data concerning the protein subcellular location. Some proteins annotated as plasma membrane proteins in the SWISS-PROT database, locate in outside or cytoplasmic and so on by prediction. For eight of the proteins annotated as cytoplasm, secreted or extracellular in the SWISS-PROT database, have one or more transmembrane regions according to the prediction. For example, spot 3-49, Sp-alpha, a secreted protein according to SWISS-PROT database, but has three transmembrane regions according to the prediction. Similarly, many proteins with negative GRAVY values are elucidated as integral membrane proteins with more than one potential transmembrane domain (Table S1). Similar difference between GRAVY and TMHMM was obtained in Fountoulakis M. [32] and Marmagne A. [18]. So, the sensitivity and specificity for prediction tool might have been overevaluated previously. Therefore, results derived from prediction should be considered with caution.

A systematic study of Arabidopsis thaliana plasma membrane proteins demonstrated that substantial part of putative plasma membrane proteins is peripheral and multilocation proteins [37]. For instance, these authors detected an actin depolymerizing factor that was localized both in the cytosol and at the PM. 7 proteins that locate in other subcellular membrane should be reexamined. Some of them have high scores compared with the abundant authentic PM proteins, suggesting that these proteins are intrinsic PM proteins rather than contaminating ones. Of these, Cytochrome c1 (spot 213), and peroxisomal bifunctional enzyme belong to the abundant proteins in the organelles. It may not be surprising to find such proteins in PM.

Fig.9 displays the function distribution of the identified membrane proteins. About 40% of the identified proteins of the mouse liver plasma membrane are enzyme subunits with a broad spectrum of catalytic activities, consistent with the fact that most enzymes focusing on PM are generally expressed at high and constitutive levels. It should also be noticed that hypothetical proteins and proteins with unclassified or unknown function represent 9% of the identified proteins. We identified 18 proteins that of unknown function in expasy databases with the method of 2DE-MS. Of which, 4 genes were found to be mapped within chromosome regions linked to 4 of liver disease such as Limb-Girdle Muscular Dystrophy 2B, Juvenile Amyotrophic Lateral Sclerosis with the use of Genbank's human genome resources (Diseases mapped to large chromosome regions were obtained from www.neuro.wustl. Edu/neuromuscular (Table 2).)

In summary, we applied proteomics technologies to analyze a standard preparation of mouse liver plasma membrane. Through comparing three kinds of lysis buffers, we found it was better to extract membrane proteins combining zwitterion detergent (CHAPS) and non-ion detergent (NP-40). In the plasma membrane fraction, we identified 175-gene products unambiguously with 2DE-MS. Approximately one third of the proteins were predicted to be

plasma membrane proteins, and plasma membrane–associated, carrying one to eight transmembrane domains. For 55% of the proteins, no annotation about their subcellular location existed in the SWISS-PROT database. About 23% of the annotated proteins of this study are PM proteins. Our results showed that some identified proteins are predicted to be cytosolic proteins and a few other subcellular proteins. The main reason is that it is difficult to get very pure plasma membrane free of other subcellular contamination. Up to date, most of the reports about PM proteomics used the density grade centrifugation to purify PM as that we did. And the contamination is the common issue in all these reports. Now some new strategies to solve this problem were proposed, such as the affinity purification [22] and subtractive proteomics [38]. These hopeful strategies still need to be improved before can be used promisingly in PM proteomics of animal tissues. Our results also show that the extraction of hydrophobic membrane proteins appears to be still difficult, and that the currently existing knowledge about protein subcellular location is limited even in well-studied organisms. It is necessary to study membrane proteins with 2DE-MS, assisted with 1DE-MS/MS. With these two methods, we identified 88 transmembrane proteins.

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Table 1 Summary of mouse liver PM transmembrane proteins identified by 2DE-MS, and 1DE-MS/MS.

Protein description	Accession	MW (Da) / pI	TMHMM	GRAVY	2DE	1DE
Myeloid upregulated protein	CAA04870	32098/8.23	8	0.718	+	
TDAG8	AAB02188	39407/8.61	7	0.206	+	
Sodium channel 3	AAA67107	49466/5.20	6	0.042	+	+
Frizzled-9 protein	AAB87503	36401/9.00	6	0.168	+	
ADP, ATP carrier protein	P51881	32931/9.74	6	0.019		+
Similar to aminopeptidase N	XP_355626	120381/8.56	6	0.144		+
Unnamed protein product	BAB26162	30107/5.4	6	0.144		+
ZnT-1	AAA79233	54716/6.20	5	0.053	+	+
I1-12 receptor beta2	CAA59167	98197/7.98	4	-0.321	+	
Integrin beta3 subunit	AAB94086	86695/5.14	3	-0.269	+	
Sp-alpha	AAB70571	38863/5.01	3	0.7181	+	
Class Ib MHC antigen Qa-2 precursor	AAB41657	37642/5.62	3	-0.355		+
H-2 class I histocompatibility antigen Q8 α-chain precursor	B24582	37330/5.52	3	-0.333		+
Cyritestin	CAA45534	91389/5.57	3	-0.302	+	

MHCH-2Dr	AAA39605	40540/5.97	2	-0.434	+	
Type II transforming growth factor-beta receptor	AAB30100	64576/6.02	2	-0.335	+	
MB2w3	CAA62323	28860/6.28	2	0.024	+	
Biliary glycoprotein	CAA53699	29984/9.22	2	-0.007	+	
H-2 class I histocompatibility antigen pH-2D-24	A21198	40148/7.7	2	-0.447		+
Glucuronosyltransferase Ugt1.6	A55788	61017/8.91	2	-0.004		+
Keratinocyte growth Factor receptor	AAA39377	79656/6.40	2	-0.342	+	
Myelin associated-glycoprotein	AAA39487	64268/5.00	2	-0.056	+	
MHC H2-K antigen	AAA39652	41758/6.22	2	-0.373		+
H2-K gene product	AAA80453	41603/6.02	2	-0.375		+
Wnt 10a	AAB08085	46454/9.30	2	-0.452	+	
Pactolus;integrin beta subunit-like cell-surface protein	AAC25502	81606/6.68	2	-0.308	+	
Semaphorin M-sema G	AAC52964	95715/8	2	-0.281	+	
Ugt2b5 protein	AAH28262	61404/7.94	2	-0.036		+
UDP glycosyltransferase 1 family polypeptide A2	AAP48594	60957/8.3	2	0.075		+
UDP glycosyltransferase 1 family polypeptide A5	AAP48595	60686/8.33	2	0.044		+
UDP glycosyltransferase 1 family polypeptide A7	AAP48596	61025/8.91	2	0.016		+
UDP glycosyltransferase 1 family polypeptide A9	AAP48597	61018/8.91	2	-0.010		+

UDP glycosyltransferase 1 family polypeptide A10	AAP48598	60529/9.01	2	-0.003		+
UDP glycosyltransferase 1 family polypeptide A13	AAP48600	61130/8.4	2	0.036		+
Pheno 1 UDPglucuronosyltransferase	BAA13483	60992/8.92	2	0.001		+
Unnamed protein product	BAC87656	60460/8.64	2	0.042		+
Unknown	CAA07480	93011/5.50	2	-0.525	+	
H-2K (d) antigen	CAA25956	41750/6.02	2	-0.374		+
DELTA-like 1	CAA56865	78449/5.90	2	-0.449	+	+
E-selectin ligand-1	CAA58855	133735/6.45	2	-0.495	+	
UDPglucuronosyltransferase 2 family, member 5	NP_033493	61386/7.94	2	-0.031		+
UDPglucuronosyltransferase 1 family, member 2	NP_038729	60987/8.3	2	0.074		+
UDP glycosyltransferase 1 family, polypeptide A6	NP_659545	61026/8.84	2	-0.002		+
Expressed sequence AI788959	NP_705826	61351/7.96	2	-0.007		+
semaphorin VIa	O35464[a]	99077/7.90	2	-0.319	+	
H-2 class I histocompatibility antigen, k-d alpha chain precursor (H-2K (d))	P01902	41749/6.16	2	-0.374		+
UDPglucuronosyltransferase 1-7 precursor	Q62452	59896/8.64	2	0.046		+
Similar to H-2 class I histocompatibility antigen, q8 α-chain precursor	XP_207059	35189/5.35	2	-0.452		+
ADAM 4 protein precursor	AAA74922	51795/6.64	1	-0.257	+	+

Epsilon tyrosine phosphatase	AAC52281	80628/6.70	1	-0.358	+	
Interleukin-4 receptor precursor	AAA39300	26456/5.57	1	-0123	+	
Meprin beta-subunit	AAA75234	79549/5.59	1	-0.332	+	
MSG2 gamma salivary protein	AAB93512	16158/6.89	1	-0.376	+	
tyrosinase	BAA00340	31121/5.22	1	-0.252	+	
TL antigen	CAA26859	40198/5.39	1	-0.579	+	
Major histocompatibility complex HLA I	1401243A	39581/6.09	1	-0.467		+
Cytochrome P450 2D10	A30247	57554/6.16	1	-0.088		+
dnaK-type molecular chaperone	A37048	72491/5.12	1	-0.48	+	
grp78 precursor						
Lymphoid-restricted Membrane protein	AAA21603	59588/5.05	1	-0.572	+	
T lymphocyte antigen	AAA37242	30669/8.95	1	-0.503	+	
Cyclophilin	AAA37498	22713/9.48	1	-0.146	+	
Complement protein H	AAA37759	139083/6.60	1	-0.581	+	
Secretary glycoprotein	AAA39321	41086/9.28	1	-0.360	+	
bilirubin/phenol UDPglucuronosyltransferase	AAA40524	57569/8.57	1	-0.026		+
Complement receptor CR2	AAA63295	111518/7.40	1	-0.381	+	
Testosterone 16-α-hydroxylase	AAA79023	57593/6.16	1	-0.079		+

B cell differentiation antigen Lyb-2/CD72	AAB22615	41058/6.20	1	-0.666	+	
SEK1	AAB81554	44114/8.28	1	-0.534	+	
Netrin-1	AAC52971	67740/9.10	1	-0.617	+	
NO-NAME-GIVEN	AAC53380	101403/5.50	1	-0.447	+	
Anpep protein	AAH17011	110721/5.62	1	-0.259		+
Ugt1a1 protein	AAH19434	57495/8.57	1	-0.039		+
MHC class I heavy chain maturation peptide H-2K (d)	AAR89506	39703/6.02	1	-0.461		+
Qa-2 cell surface antigen	BAA14174	21234/5.63	1	-0.602		+
ELM1	BAA24949	40703/6.74	1	-0.223	+	
Mannose-binding lectin associated serine protease-2	BAA34674	75491/5.80	1	-0.220	+	
Major urinary protein	CAA26953	20694/4.90	1	-0.324	+	+
EGF-receptor	CAA42219	79302/7.70	1	-0.264	+	
Insulin-like growth factor binding protein	CAA57270	32761.9/7.45	1	-0.460	+	
Insulin-like growth factor binding protein 5	CAA57273	30372/8.50	1	-0.612	+	
Erthyrocyte band 7 integral mebrane protein, protein 7.2B, stomatin	CAA62503	31441/6.50	1	0.059	+	

Cytochrome P450, family2, subfamily f, polypeptide 2	NP_031843	56139/7.74	1	-0.144		+
Alanyl aminopeptidase	NP_032512	110022/5.62	1	-0.272		+
Dipeptidylpeptidase 4	NP_034204	88065/5.96	1	-0.323		+
Endoplasmin precursor	P08113	92703/4.74	1	-0.720	+	
Cytochrome P450 2F2	P33267	56141/7.74	1	-0.135		+
FK506 binding protein 14 precursor	P59024	24350/5.66	1	-0.428	+	
Dipeptidyl-peptidase IV α-chain	S23752	88093/5.96	1	-0.320		+

Protein database accession numbers is protein ID. In the columns pI and MW, the theoretical pI and molecular mass values are listed. The "GRAVY" and "TMHMM" are listed (predicted through http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html).

Table 2 Possible associations of previously unknown PM proteins with genetic diseases.

Protein name	Accession	Accession (Human homolog protein)	Chrom location	Disease-linked chromosome regions	Diseases
Unknown protein	CAA07480	gi|14423632|sp|P78536|AD17	2p25	2p	Limb-Girdle Muscular Dystrophy 2B
NO-NAME-GIVEN	AAC53380	gi|41872567|ref|NP_957719.1	2q33.3	2q33-q35	Juvenile Amyotrophic Lateral Scierosis
Unnamed protein product	BAB22380	ref|NP_001907.2	8q24.3	8q	Charcot-Marie-Tooth Disease 2A
NO-NAME-GIVEN	CAA40341	gi|4885281|ref|NP_005262.1	10q23.3	10q22-q24	Isolated dilated cardiomyopathy

Figures and Legends

Fig. 1 Electron micrographs of mouse liver plasma membrane.

(A) Scanning electron microscope, x600. (B) Transmission electron microscope, x50,000.

Fig.2 Silvered-stained 2-DE maps of plasma membrane proteins.

Mouse liver PM proteins extracted by different lysis buffers and procedures were separated over a first-dimension 18 cm IPG strip pH 3-10, followed by a second-dimension 20cm SDS-PAGE. (A) The whole plasma membrane proteins solubilized with Lysis buffer 1, T=10%, 532 spots detected; (B) 0.1M Na_2CO_3 treated PM proteins solubilized with Lysis buffer 1, T=10%, 280 spots detected (C) $CHCl_3/CH_3OH$ (5:4v/v) treated PM proteins solubilized with Lysis buffer 1, T=11.5%, 232 spots detected; (D) Triton X-114 treated PM proteins solubilized with Lysis buffer 1, T=11.5%, 36 spots detected. (E); 0.1M Na_2CO_3 treated PM proteins solubilized with Lysis buffer 2, T=10%, 271 spots detected (F) 0.1M Na_2CO_3 treated PM proteins solubilized with Lysis buffer 3 T=10%, 251 spots detected

Fig.3 2-DE analysis of the plasma membrane proteins extracted with lysis buffer 1.

The membrane proteins were solubilized with lysis buffer 1 (8M urea, 2M thiourea, 4%(w/v) CHAPS and 1%(v/v) NP-40) and separated in a pH 3-10 linear IPG strip, followed by an 11.5% SDS-polyacrylamide gel, as stated under Material and methods. The gel was stained with Coomassie blue. The proteins were identified by MALDI-TOF, ESI-Q-TOF or Western blot and are designated with the number. The proteins are listed in Table S1.

Fig.4 2-DE analysis of the plasma membrane proteins extracted with lysis buffer 2.

The membrane proteins were solubilized with Lysis buffer 2 (7M urea, 2M thiourea, and 1% ASB-14 (w/v)) and separated in a pH 3-10 linear IPG strip, followed by a 13.5% SDS-polyacrylamide gel, as stated under Material and methods. The gel was stained with Coomassie blue. The proteins were identified by MALDI-TOF, or ESI-Q-TOF and are designated with the number. The proteins are listed in Table S1.

Fig. 5 2-DE analysis of the plasma membrane proteins extracted with lysis buffer 3.

The membrane proteins were solubilized with lysis buffer 3 (5M urea, 2M thiourea, and 2% CHAPS (w/v) and 2%(w/v) SB3-10) and separated in a pH 3-10 linear IPG strip, followed by an 11.5% SDS-polyacrylamide gel, as stated under Material and methods. The gel was stained with Coomassie blue. The proteins were identified by MALDI-TOF, or ESI-Q-TOF and are designated with the number. The proteins are listed in Table S1.

Fig. 6 2-DE analysis of the plasma membrane proteins treated with 0.1%Na_2CO_3.

The membrane proteins were solubilized with Lysis buffer 1 (8M urea, 2M thiourea, 4%(w/v) CHAPS and 1%(v/v) NP-40) and separated in a pH 3-10 linear IPG strip, followed by an 11.5% SDS-polyacrylamide gel, as stated under Material and methods. The gel was stained with Coomassie blue. The proteins were identified by MALDI-TOF and are designated with the number. The proteins are listed in Table S1.

Fig. 7 MALDI-TOF mass spectrum of spot 1N

The sample was deposited onto a 96-well target plate, and the solvent was removed by air-drying at room temperature. The peptide mixture was analyzed by MALDI-TOF-MS.

Fig. 8 MS/MS mass spectrum of the doubly charged ion with *m/z* 852.1 of a peptide (GLGTDSILNLLTSR) eluting from the nano LC run of the in situ digest of annexin V.

Fig. 9 The function distribution of the mouse liver PM proteins

Fig.10 The hydrophobic distribution of membrane proteins identified by 2DE-MS.

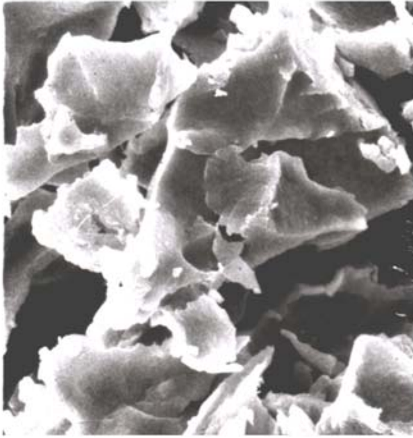
Positive GRAVY scores show hydrophobic proteins.

Fig. 11 Mouse liver PM proteins separated by 1-DE.

200μg per lane (lane 1,2,3 and 4) of PM proteins extracted by lysis bufer1 was separated by SDS-PAGE and stained with Coomassie blue. M, mass weight of markers. The protein bands marked with number were digested and identified by ESI-MS/MS.

Fig. 1

A



B

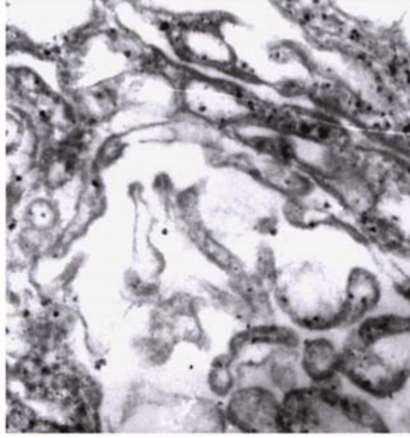


Fig. 2

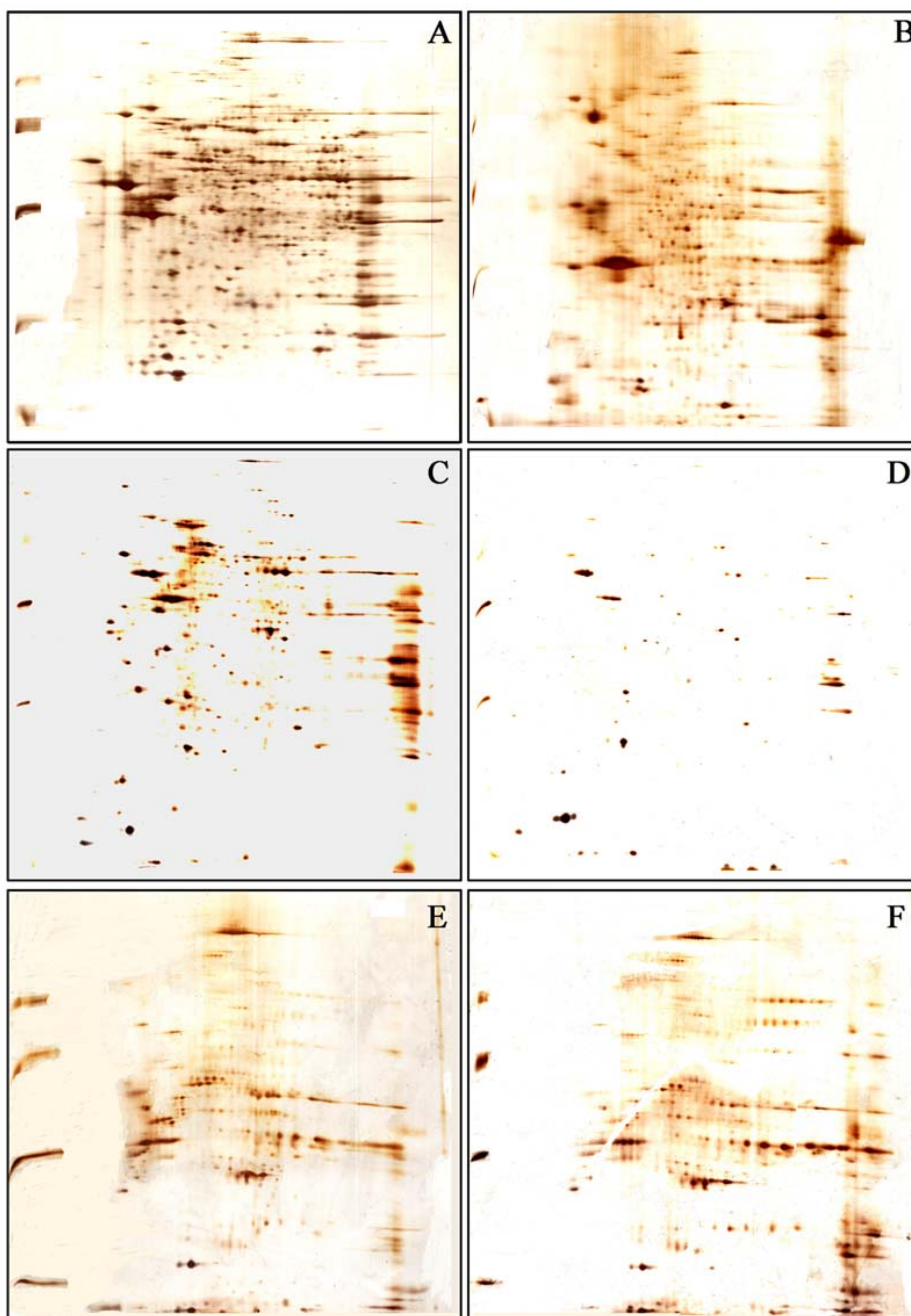
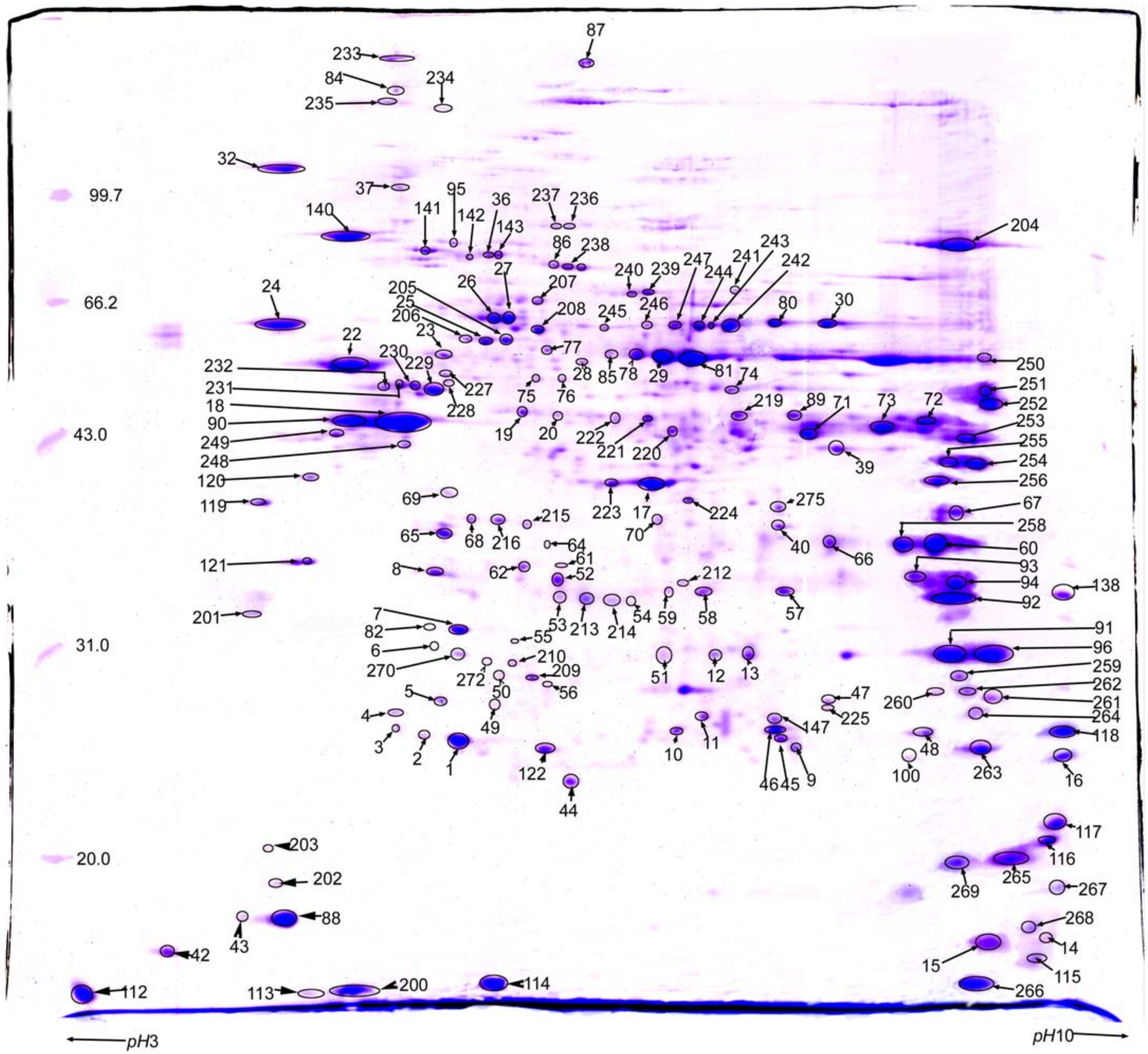


Fig. 3



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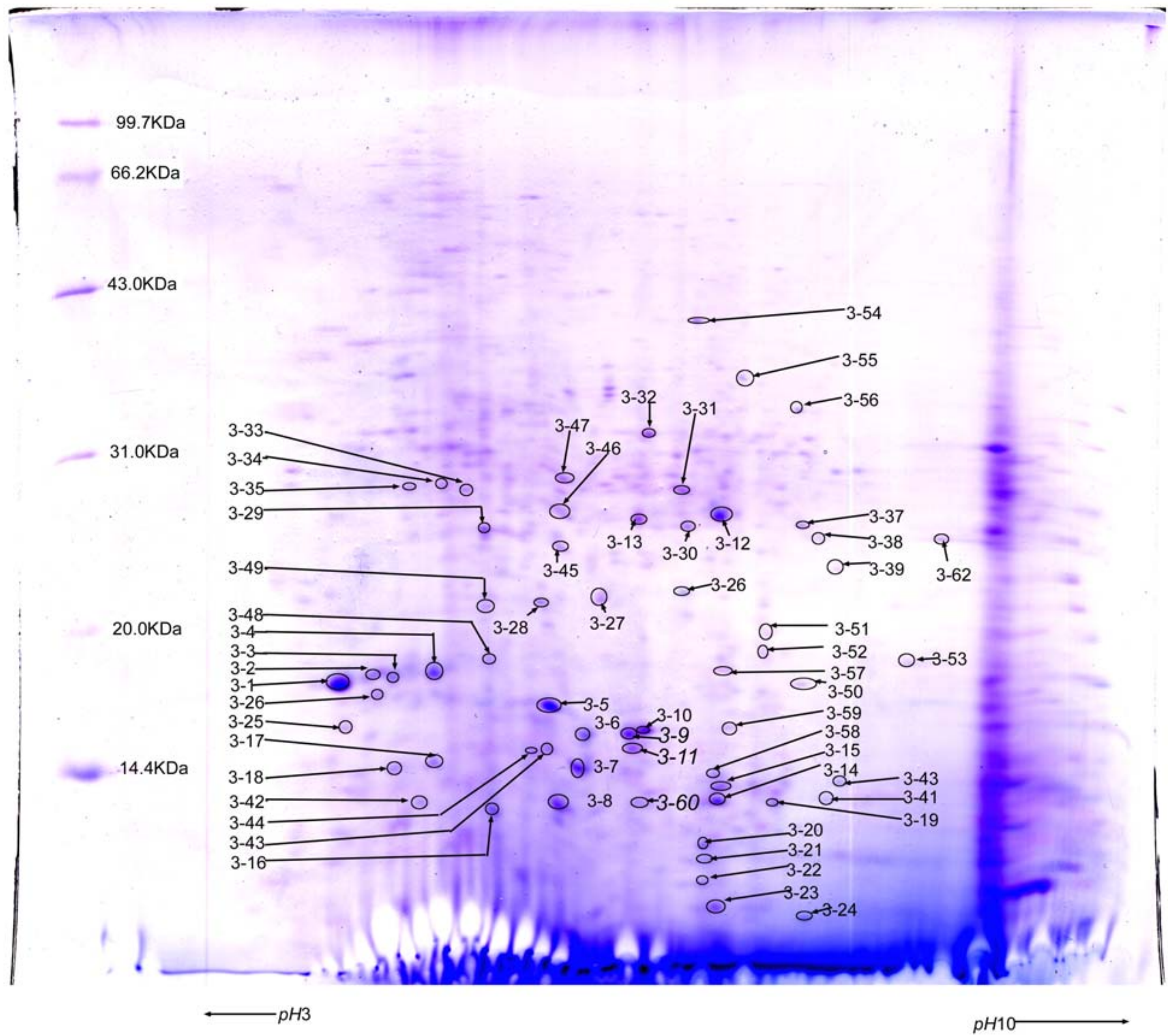


Fig.5

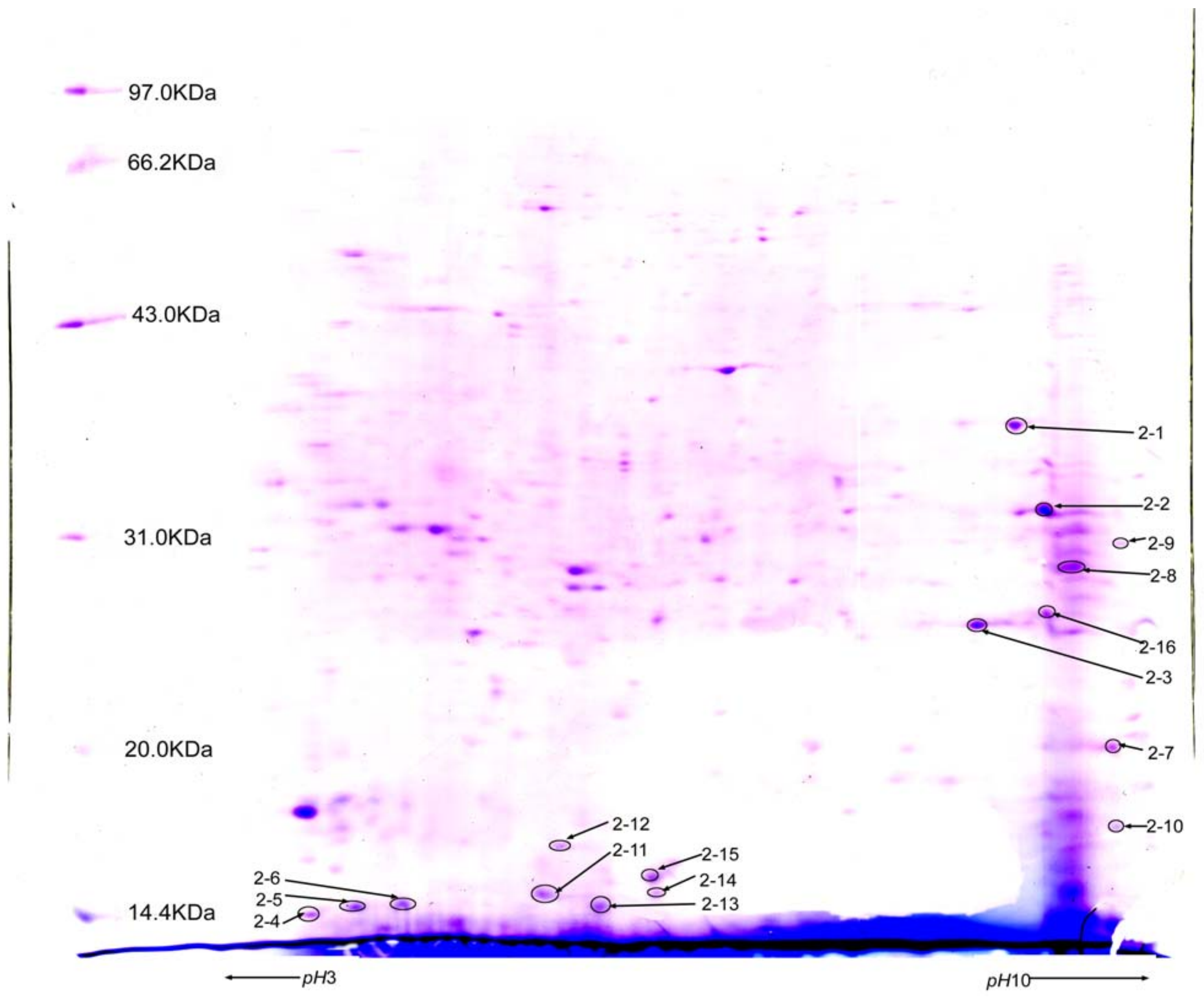


Fig.6

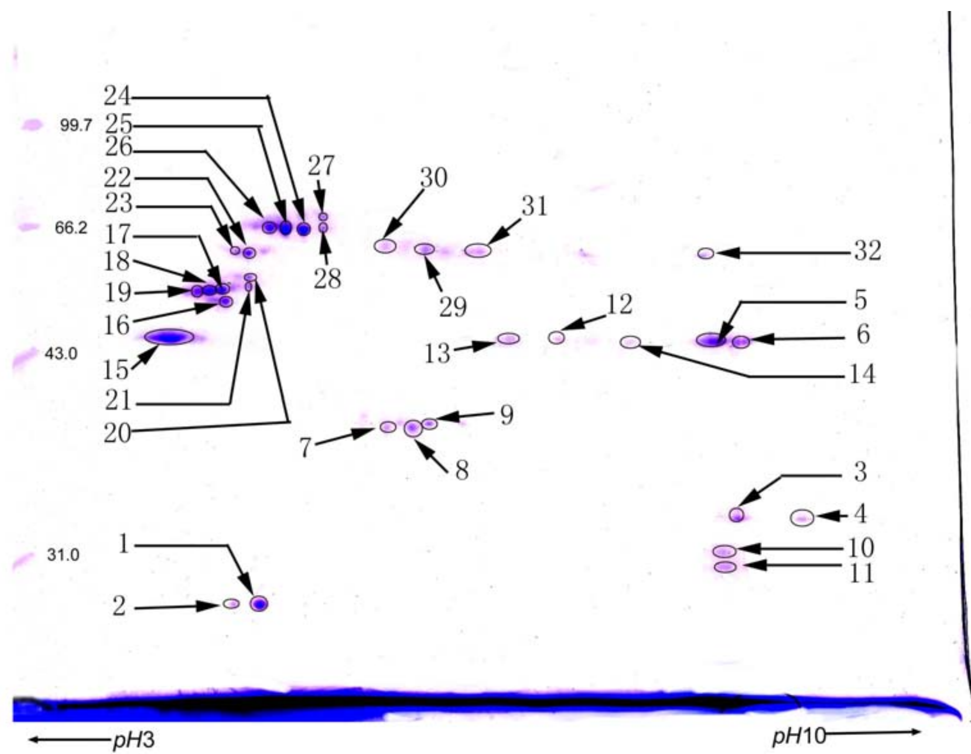


Fig.7

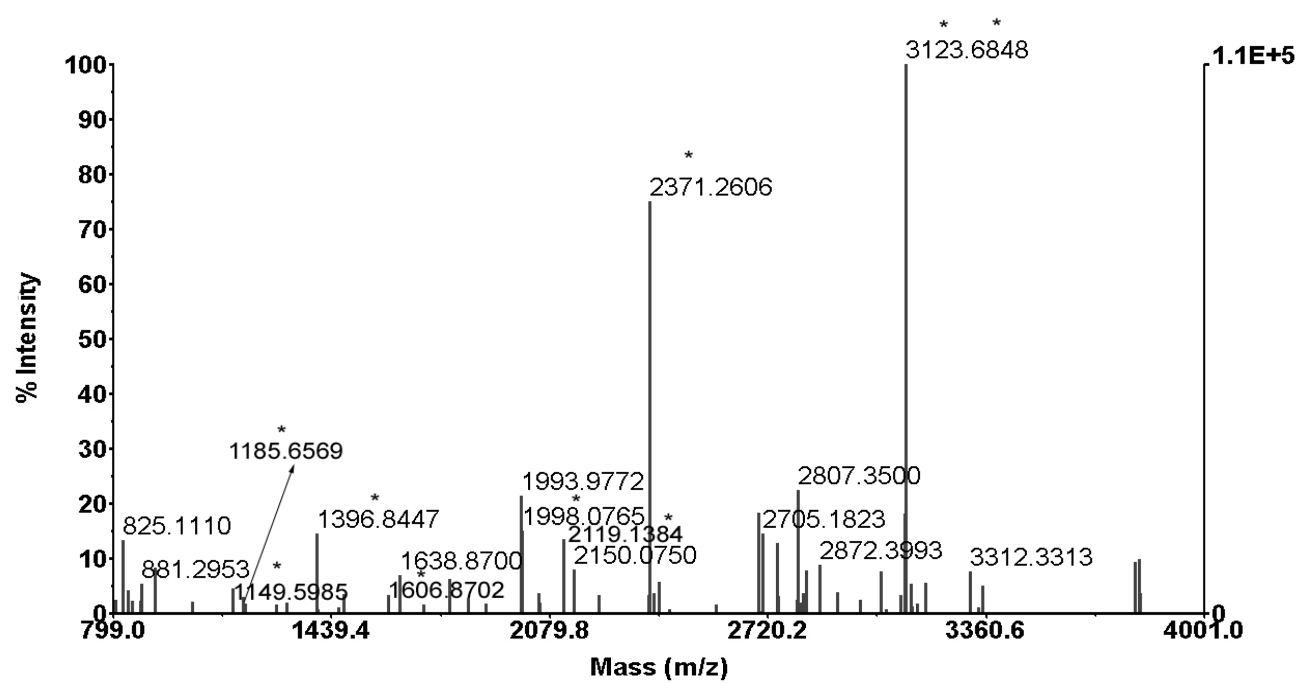


Fig.8

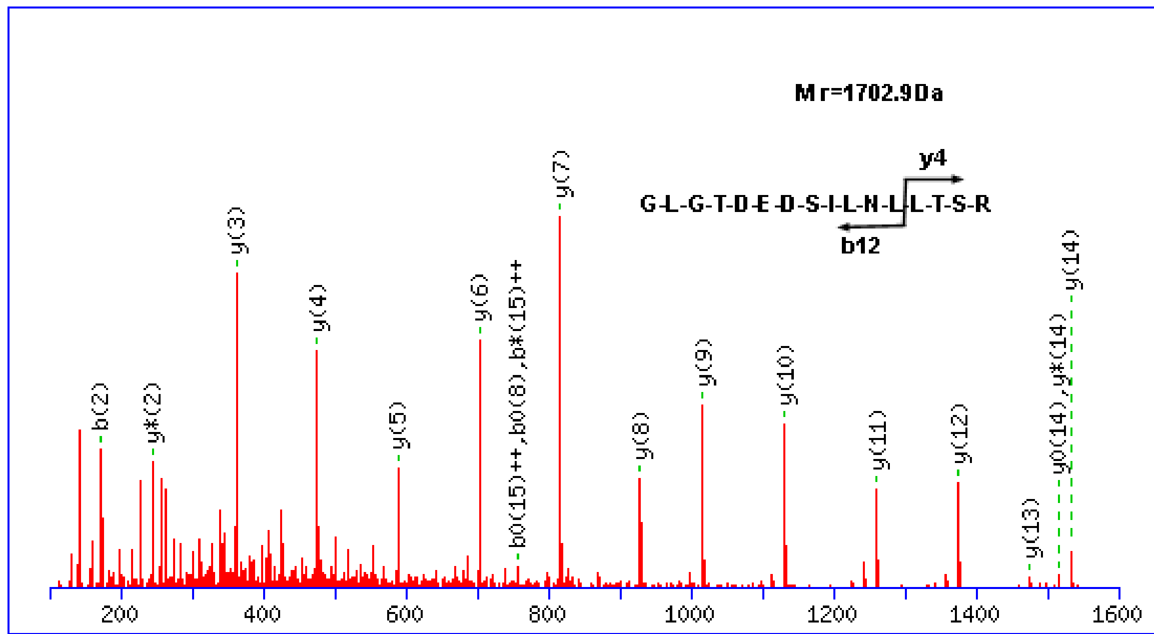


Fig.9

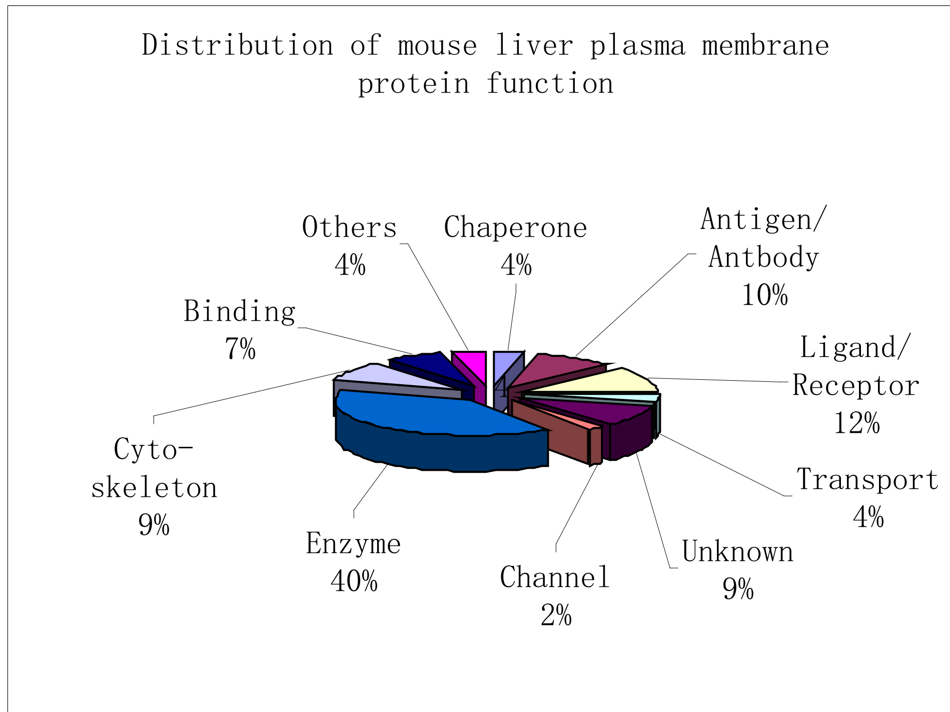


Fig. 10

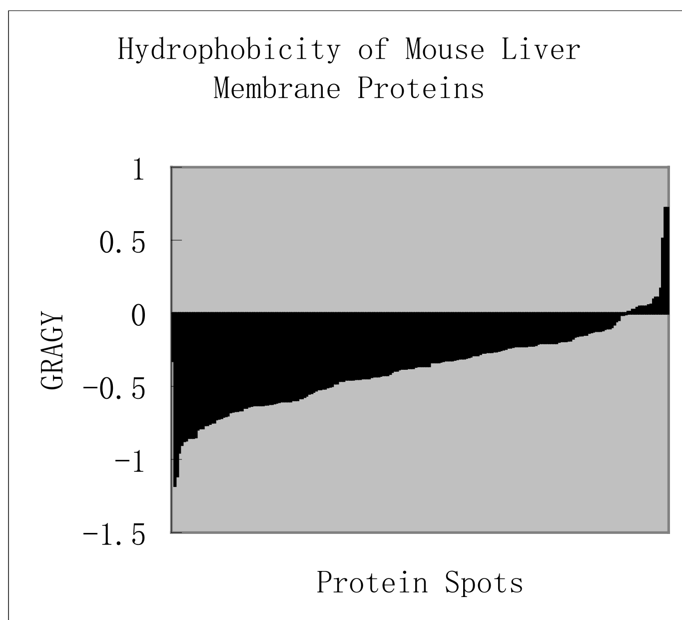
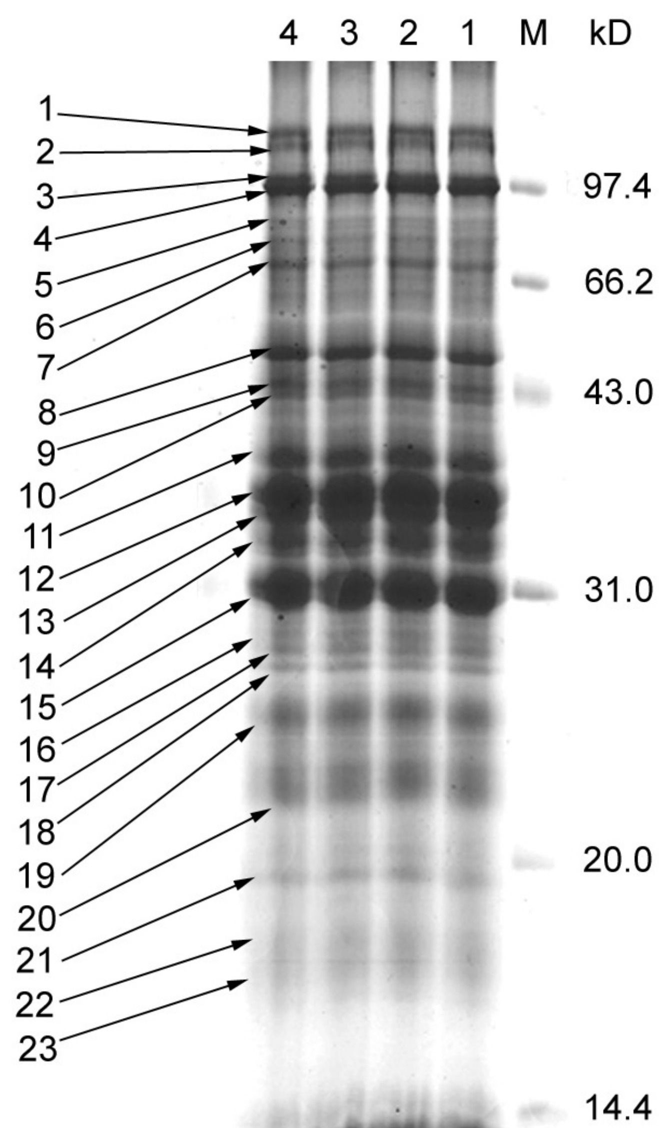


Fig. 11



Supporting online table

Table S1 Mouse liver PM proteins separated from 2DE

Spot Number	Protein description	Accession	MW (Da) / pI	Function	Cover	Subcellular Location (predicted)	TMHMM	GRA
1	Major urinary protein	CAA26953	20694/4.90	Antigen	31%	Secreted (Outside)	1	-0.32
88	Major urinary protein	CAA26953	20694/4.89	Antigen	35%	Secreted (Outside)	1	-0.32
2	Beta-tropomyosin	AAA40484	32837/4.70	Cytoskeleton	16%	(Cytoplasm)	0	-1.11
3	Lymphoid-restricted Membrane protein	AAA21603	59588/5.05	Cytoskeleton	30%	PM (pm)	1	-0.57
5	Erthyrocyte band 7 integral mebrane protein, protein 7.2B,	CAA62503	31441/6.50	Enzyme	22%	(PM)	1	0.059

	stomatin							
6	Keratinocyte growth Factor receptor	AAA39377	79656/6.40	Receptor	18%	Type I membrane protein (PM)	2	-0.34
7	32	CAA55349	29820/5.60	Receptor	48%	(ER)	0	0.009
1N	32	CAA55349	29820/5.57	Receptor	49%	(ER)	0	0.009
2N	32	CAA55349	29820/5.57	Receptor	53%	(ER)	0	0.009
8	Apolipoprotein E	AAA37252	33227/5.80	Enzyme	41%	(Cytoplasm)	0	-0.89
9	DELTA-like 1	CAA56865	78449/5.90	Antigen	20%	Type I membrane protein (PM)	2	-0.44
49	DELTA-like 1	CAA56865	78449/5.90	Antigen	19%	PM (PM)	2	-0.44
10	Acidic epididymal Glycoprotein Like protein	AAA37186	27315/8.70	Unknown	20%	(Outside)	0	-0.31
11	Netrin-1	AAC52971	67740/9.10	Enzyme	17%	Extracellular (outside)	1	-0.61
253	Netrin-1	AAC52971	67740/9.10	Enzyme	20%	Extracellular (outside)	1	-0.61
12	Keratin D	AAA39373	47489/5.30	Cytoskeleton	28%	PM (MMS)	0	-0.62

13	Ajuba;jub	AAB38287	57902/7.10	Metal-binding	18%	(MMS)	0	-0.42
14	Protein-tyrosine-phosphatase	CAA60477	86992/5.81	Enzyme	18%	PM (MMS)	0	-0.79
15	Wnt 10a	AAB08085	46454/9.30	Ligand	20%	PM (Outside)	2	-0.45
3-23	Wnt-3 protein	AAB38109	39659/7.47	Ligand	24%	(Outside)	0	-0.36
16	Keratin 10	AAA59199	39742/4.70	Cytoskeleton	18%	(Cytoplasm)	0	-0.70
17	Complement protein H	AAA37759	139083/6.60	Cofactor	18%	Secreted (Outside)	1	-0.58
3-47	Alpha-cardiac actin	AAA37167	41785/5.23	Cytoskeleton	16%	(Cytoplasm)	0	-0.24
18	Gamma-actin	CAA31455	41019/5.60	Cytoskeleton	55%	(Cytoplasm)	0	-0.20
69	Gamma-actin	CAA31455	41019/5.60	Cytoskeleton	24%	(Cytoplasm)	0	-0.20
248	Gamma-actin	CAA31455	41019/5.56	Cytoskeleton	19%	cytoplasmic	0	-0.20
249	Gamma-actin	CAA31455	41019/5.56	Cytoskeleton	35%	cytoplasmic	0	-0.20
2-15	Gamma-actin	CAA31455	41019/5.56	Cytoskeleton	36%	cytoplasmic	0	-0.20
3-34	Gamma-actin	CAA31455	41019/5.56	Cytoskeleton	35%	(Cytoplasm)	0	-0.20
3-35	Gamma-actin	CAA31455	41019/5.56	Cytoskeleton	36%	(Cytoplasm)	0	-0.20
3-11	Gamma-actin	P02571 [a]	42167/5.47	Cytoskeleton	26%	Cytoplasmic	0	-0.20

						(Cytoplasm)		
3-11[E]	Actin, cytoplasmic 2	P02571[a]	42167/5.47	Cytoskeleton	33%	Cytoplasmic (Cytoplasm)	0	-0.20
19[E]	Similar to NADH dehydrogenase (ubiquinone) Fe-s protein 2	Q91WD5[a]	52991/6.52	Enzyme	23%	(Mitochondrial)	0	-0.28
3-2	Keratin	P11679	54284/5.50	Cytoskeleton	15%	(Secreted)	0	-0.44
20	Dynamin	AAA37318	85041/6.00	Transport	20%	Cytoplasmic (Cytoplasm)	0	-0.51
21	I1-12 receptor beta2	CAA59167	98197/7.98	Receptor	18%	(PM)	4	-0.32
22	ATP synthase beta-subunit	AAB86421	56380/5.10	Enzyme	56%	cytosolic (MMS)	0	0.044
23	ATP synthase beta-subunit	AAB86421	56380/5.10	Enzyme	20%	cytosolic (MMS)	0	0.044
3-33	ATP synthase beta-subunit	AAB86421	56379.9/5.14	Enzyme	21%	cytosolic (MMS)	0	0.044
3-33[E]	ATP synthase beta chain	P56480[a]	56344/5.14	Enzyme	21%	cytosolic (MMS)	0	0.033
24	Apoptotic protease Activating factor 1	AAC62458	139782/5.90	Enzyme	15%	(Cytoplasm)	0	-0.21

25	NO-NAME-GIVEN	AAC53380	101403/5.50	Unknown	17%	(PM)	1	-0.44
26	Myelin associated-glycoprotein	AAA39487	64268/5.00	IGF-binding	18%	(PM)	2	-0.05
28	Insulin-like growth factor binding protein 5	CAA57273	30372/8.50	Enzyme	18%	Secreted (CP)	1	-0.61
257	Insulin-like growth factor binding protein	CAA57270	32761.9/7.45	Enzyme	23%	(Outside 0.82)	1	-0.46
29	TRAF 5	BAA11942	64145/7.71	Transport	25%	PM (Cytoplasm)	0	-0.56
30	B cell differentiation antigen Lyb-2/CD72	AAB22615	41058/6.20	Antigen	18%	PM (PM)	1	-0.66
32	Mannose-binding lectin associated serine protease-2	BAA34674	75491/5.80	Enzyme	17%	(Outside)	1	-0.22
34	Heat shock 73 protein	AAC52836	70871/5.40	Chaperone	31%	(Necleus)	0	-0.45
36	Alpha-fetoprotein	AAA37190	47226/5.50	Estrogens-binding	15%	Secreted	0	-0.41
37	semaphorin VIa	O35464	99077/7.90	Ligand	25%	Type I membrane protein	2	-0.31

						(Outside)		
38	Angiopoietin-2	AAB63189	56617/5.40	Ligand	21%	Secreted. (Cytoplasm)	0	-0.60
39	Hi-calponin alpha	AAB01453	33329/8.80	Actin-binding	19%	(Cytoplasm)	0	-0.78
40	Arginase	AAA98611	34808/6.50	Enzyme	34%	Cytoplasm (Nucleus)	0	-0.18
9 N	Arginase	AAA98611	34808/6.51	Enzyme	15%	(Nucleus)	0	-0.18
3-54	Arginase	AAA98611	34808/6.51	Enzyme	46%	Cytoplasmic (Necleus)	0	-0.18
42	Collagen	AAA37346	12420/6.90	Cytoskeleton	20%	(cytosome)	0	-0.63
43	ELM1	BAA24949	40703/6.70	Transport	19%	(PM)	0	-0.22
250	ELM1	BAA24949	40703/6.74	Transport	16%	Cytoplasmic (PM)	1	-0.22
26 N	ELM1	BAA24949	40703/6.74	Transport	25%	(PM)	1	-0.22
3-56	ELM1	BAA24949	40703/6.74	Transport	23%	Cytoplamic Translocation to plasma membrane (PM)	1	-0.22
44	Retinal binding protein	AAB06955	23206/5.70	Receptor	45%	(Outside)	0	-0.51

45	OSF-3	BAA04796	22177/8.30	Enzyme	19%	PM (microbody)	0	-0.22
48	Frizzled-9 protein	AAB87503	36401/9.00	Receptor	25%	(PM)	6	0.168
50	Antioxidant enzyme AOE372	AAB57846	31053/6.70	Enzyme	39%	(microbody)	0	-0.26
51	Phospholipase C-alpha	AAA39944	56622/6.00	Enzyme	18%	(Outside)	0	-0.47
52	Ubiquitin protein ligase	AAB99764	97975/6.08	Enzyme	20%	(Microbody)	0	-0.66
53	Fibrillin-1	AAA61825	118504/5.30	Ca^{2+}-binding	15%	(Cytoplasm)	0	-0.37
240	Fibrillin-1	AAA61825	118504/5.30	Ca^{2+}-binding	15%	(Cytoplasm)	0	-0.37
54	Zinc finger protein	AAA67545	62612/9.20	Cytoskeleton	23%	(PM)	0	-0.95
55	Semaphorin M-sema G	AAC52964	95715/8	Immune	15%	Type I membrane protein (PM)	2	-0.28
56	1-Cys peroxiredoxin	AAC63376	24827/6.00	Enzyme	23%	Cytoplasmic (Microbody)	0	-0.22
57	Voltage dependent anion Channel 2	AAC13321	31733/7.40	Transport	39%	PM (microbody)	0	-0.22
58	Voltage dependent anion	AAC13321	31733/7.40	Transport	45%	PM (microbidy)	0	-0.22

	Channel 2							
60	NO-NAME-GIVEN	AAA40538	35039/8.50	Unknown	51%	(Microbody)	0	-0.46
258	Unknown	AAA40538	35039/8.48	Unknown	52%	(Microbody)	0	-0.46
2-2	No name given	AAA40538	35039/8.48	Unknown	39%	(Microbody)	0	-0.46
61	Sphingosine kinase	AAC61697	55120/6.40	Enzyme	20%	PM (Cytoplasm)	0	-0.11
62	NST-1	AAA17724	54986/5.60	ATP binding	14%	(Cytoplasm)	0	-0.11
64	Cytosolic malate dehydrogenase	AAA37423	36477/6.20	Enzyme	31%	(PM)	0	-0.05
65	Lipoxygenase	AAC79681	80586/6.20	Enzyme	17%	PM (Nuclear)	0	-0.20
66	ZnT-1	AAA79233	54716/6.20	Transport	16%	Integral membrane protein. Located on the plasma membrane (PM)	5	0.053
67	Glyceraldehyde-3-phosphate dehydrogenase	AAA37659	35810/8.40	Enzyme	20%	PM (MMS)	0	-0.09
70	EGF-receptor	CAA42219	79302/7.70	Receptor	17%	(PM)	1	-0.26

245	EGF-receptor	CAA42219	79317/7.68	Receptor	19%	(PM)	1	-0.26
72	Cytokeratin	AAA37552	47538/5.20	Cytoskeleton	17%	(MM)	0	-0.62
73	KIFC1	BAA19676	66205/9.30	ATP binding	18%	(Cytoplasm)	0	-0.50
74	ICAp69	AAC52992	54383/5.60	Antigen	16%	(Cytoplasm)	0	-0.67
76	SH2-containing inositol -phosphatase	AAB18937	133414/7.80	Enzyme	16%	(Cytoplasm)	0	-0.54
77	Aldehyde dehydrogenase	AAA64636	56538/7.50	Enzyme	41%	Cytoplasmic (MMS)	0	-0.12
78	No name given	CAA40341	61337/8.05	Unknown	48%	(MMS)	0	-0.30
80	ATP synthase alpha subunit	AAA37271	59753/9.22	Enzyme	20%	(MMS)	0	-0.10
30 N	ATP synthase alpha subunit	AAA37271	59753/9.22	Enzyme	18%	(MMS)	0	-0.10
81	Osteonectin	AAA40125	34450/4.77	Ca^{2+}-binding	25%	PM (Outside)	0	-0.42
85[E]	Type I cytoskeletal 18	P05784[a]	47329/5.28	Cytoskeleton	21%	(MMS)	0	-0.62
119[E]	Keratin, type I cytoskeletal 18(Cytokeratin 18)	P05784[a]	47344/5.22	Cytoskeleton	31%	(MMS)	0	-0.62
231[E]	Cytokeratin 18	P05784[a]	47344/5.22	Cytoskeleton	19%	(Mitochondrial)	0	-0.62

86	Acetylcholinesterase	CAA39867	68169/5.90	Enzyme	22%	Occur on the outer surface of cell membrane (lysosome)	0	-0.15
87	E-selectin ligand-1	CAA58855	133735/6.45	Ligand	18%	(PM)	2	-0.49
91	NIPSNAP 1 protein	CAA04634	33363/9.48	Unknown	19%	(ERM)	0	-0.62
96	NIPSNAP1 protein	CAA04634	33363/9.48	Unknown	45%	(ER)	0	-0.62
262	NIPSNAP 1 protein	CAA04634	33363/9.48	Unknown	27%	(ER)	0	-0.62
92	Sodium channel 3	AAA67107	49466/5.20	Channel	22%	Integral membrane protein (pm0.64)	6	0.042
93	Voltage dependent annion channel 1	AAB47777	30756/8.62	Channel	36%	PM (microbody)	0	-0.42
94	Voltage dependent annion channel 1	AAB47777	30756/8.62	Channel	36%	PM (microbody)	0	-0.42
95	P66 mot1	BAA01862	73529/5.91	Chaperone	45%	(MMS)	0	-0.39
112[E]	cytokeratin KRT2-6HF	AAK17206	42357/5.11	Cytoskeleton	6%	(Cytoplasm)	0	-0.62
114	FK506 binding protein 14	P59024	24350/5.66	Enzyme	28%	Endoplasmic reticulum	1	-0.42

	precursor					lemen (Outside)		
115	Thymopoietin alpha	AAC52578	75332/8.60	Protein binding	17%	Nuclear membrane (Cytoplasm)	0	-0.59
116	nicein	AAA68093	50860/8.56	Receptor	18%	PM	0	-0.62
117[E]	Hypothetical 19.0 KDa protein	Q8VC94 [a]	19240/9.82	Cytoskeleton	8%	(ERM)	0	-0.43
118	Ubiquinol-cytochrome C reductase complex, subunit VI requiring protein	AAB98530	19576/10.31	Enzyme	21%	(Cytoplasm)	0	-0.45
120[E]	Keratin, type II cytoskeletal 8(cytoskeletal 8)	P11679 [a]	54428/5.70	Cytoskeleton	20%	(Mitochondrial intermembrane space)	0	-0.60
205[E]	Type II cytoskeletal 8	P11679 [a]	54428/5.70	Cytoskeleton	9%	(Mitochondrial intermembrane space)	0	-0.60
227[E]	Type II cytoskeletal 8	P11679 [a]	54284/5.50	Cytoskeleton	3%	(Mitochondrial intermembrane space)	0	-0.60
229[E]	Type II cytoskeletal 8	P11679 [a]	54284/5.50	Cytoskeleton	9%	(Mitochondrial	0	-0.60

						intermembrane space)		
122[E]	Thioredoxin peroxidase 2	P35700[a]	22390/8.26	Enzyme	16%	(Mitochondrial space)	0	-0.22
121	annexin V	BAA09728	35753/4.83	Channel	26%	PM (Cytoplasm)	0	-0.33
201[E]	Annexin A5	P48036[a]	35787/4.83	Channel	46%	PM (Cytoplasm)	0	-0.33
122	Complement receptor CR2	AAA63295	111518/7.40	Receptor	16%	Type I membrane protein (PM0.7)	1	-0.38
140[E]	dnaK-type molecular chaperone grp78 precursor	A37048	72491/5.12	Chaperone	24%	(ER0.91)	1	-0.48
141	Heat shock cognate 71Kda protein	P08109[a]	71055/5.37	Chaperone	9%	(Nucleus)	0	-0.45
142[E]	Heat shock cognate 71Kda protein	P08109[a]	71055/5.37	Chaperone	13%	(Nucleus)	0	-0.45
143[E]	Unnamed protein product	BAC34360	42814/6.47	Unknown	16%	(Necleus)	0	-0426
147	Receptor tyrosine kinase	AAA39521	109956/6.87	Enzyme	17%	Type I membrane protein (PM)	0	-0.31

204[E]	Peroxisomal bifunctional enzyme (Enoyl-CoA hydratase)	Q9DBM2 [a]	78633/9.26	Enzyme	35%	Peroxisomal (By similarity) (Cytoplasm)	0	-0.11
206[E]	Munendoaa	AAA37551	53210/5.42	Unknown	12%	(Mitochondrial intermembrane space)	0	-0.60
207	Carboxylesterase , Es-male	AAB27606	61510/5.89	Enzyme	17%	(Lysosome)	0	-0.13
208[E]	Fibrinogen	Q8K0E8[a]	55402/6.68	Binding	6%	(Outside 0.552)	0	-0.74
209[E]	Peroxiredoxin 4	O08807 [a]	31261/6.67	Enzyme	40%	Cytoplasmic (microbody)	0	-0.26
210[E]	T-complex protein 1	P80317 [a]	58424/6.63	Chaperone	8%	(Cytoplasmic)	0	-0.08
211	ECH1p	AAB84224	36119/7.60	Enzyme	23%	(Microbody)	0	-0.14
212[E]	Agmatine ureohydrolase	XP_131722	38744/8.07	Enzyme	36%	Mitochondrial (Cytoplasmic)	0	-0.07
213[E]	Cytochrome c1	Q9D0M3 [a]	35533/9.24	Transport	18%	Mitochondrial intermembrane space (Mitochondrial	0	-0.24

						intermembrane space)		
214[E]	Unnamed protein product	BAB22380	35646/9.34	Unknown	16%	(Mitochondrial)	0	-0.26
215[E]	60S acidic ribosomal protein P0	P14869[a]	34336/5.91	RNA binding	26%	(Cytoplasmic)	0	0.105
216[E]	60S acidic ribosomal protein P0	P14869[a]	34336/5.91	RNA binding	38%	(Cytoplasmic)	0	0.105
220[E]	Similar to keratin complex 2, basic, gene 6a	Q8OVP7[a]	35315/5.09	Cytoskeleton	8%	(Cytoplasmic)	0	-0.42
221	Glutamate-ammonia ligase	AAA17989	42189/6.64	Enzyme	18%	(Microbody)	0	-0.60
222[E]	Endoplasmin precursor	P08113[a]	92703/4.74	Chaperone	9%	Endoplasmic reticulum lumen (Endoplasmic reticulum lumen)	1	-0.72
224[E]	Arginase 1	NP_031508	34957/6.51	Enzyme	54%	Cytoplasmic (Necleus0.60)	0	-0.18
3-12[E]	Arginase 1	Q61176[a]	34786/6.51	Enzyme	17%	Cytoplasmic (Necleus0.60)	0	-0.18
225[E]	Argininosuccinate synthase	P16460[a]	46840/8.36	Enzyme	4%	(Cytoplasmic)	0	-0.36
230[E]	Glycogen phosphorylase	Q9WUB3[a]	97550/6.65	Enzyme	22%	(Cytoplasmic)	0	-0.40

232	Pactolus;integrin beta subunit-like cell-surface protein	AAC25502	81606/6.68	Unknown	19%	(PM)	2	-0.30
233[E]	Spectrin alpha chain	P16546 [a]	43601/5.15	Cytoskeleton	31%	PM (Cytoplasmic)	0	-0.84
234[E]	Hypothetical protein	Q8K380 [a]	156610/5.29	Calium ion binding	23%	(Neclues)	0	-0.79
235	P80 Ku autoantigen	CAA46999	83324/5.15	Antigen	21%	(Outside)	0	-0.37
27 N	P80 Ku autoantigen	CAA46999	83324/5.15	Antigen	21%	Outside	0	-0.37
236	Unknown	CAA07480	93011/5.50	Unknown	18%	(PM)	2	-0.52
237	SEK1	AAB81554	44114/8.28	Receptor	23%	(Outside)	1	-0.53
238	T-cell receptor beta chain VNDNJC precursor	AAA40243	34661/8.34	Receptor	22%	(PM)	0	-0.32
239	SKD2	AAA50498	82566/6.52	Enzyme	18%	Cytoplasmic (Cytoplasmic)	0	-0.21
241	Lymphocyte differentiation antigen	AAA39453	53849/8.60	Antigen	24%	（PM）	0	-0.38
242	Catalase	AAA66054	59734/8.10	Enzyme	34%	Peroxisomal (Cytoplasmic)	0	-0.64

244	Catalase	AAA66054	59734/8.10	Enzyme	25%	Peroxisomal (Cytoplasmic)	0	-0.63
244[E]	Catalase	P24270[a]	59851/8.09	Enzyme	15%	Peroxisomal (Cytoplasmic)	0	-0.63
3-32[E]	Catalase	P24270[a]	59851/8.09	Enzyme	16%	Peroxisomal (Cytoplasmic)	0	-0.64
243	Brevican	CAA60575	96014/4.83	Binding	19%	Secreted, extracellular matrix (Outsied)	0	-0.42
246	Secretary glycoprotein	AAA39321	41086/9.28	Unknown	21%	（Outside）	1	-0.36
247	Cyritestin	CAA45534	91389/5.57	Enzyme	16%	(PM)	3	-0.30
251	Semaphorin III	AAA73934	76453/6.72	Plays a role in growth	18%	Secreted	0	-0.66
252[E]	Enoyl coenzyme A hydratase 1	NP_803421	42260/8.33	Enzyme	3%	(MMS)	0	-0.04
254	Mitochondrial aspartate aminotrandferase precursor	AAB91426	47413/9.05	Enzyme	28%	PM (microbody)	0	-0.23
255[E]	Aspartate aminotransferase	P05202[a]	47780/9.13	Enzyme	26%	Cytoplasmic	0	-0.23

						(microbody)		
260	T lymphocyte antigen	AAA37242	30669/8.95	Antigen	20%	Type I membrane protein (PM)	1	-0.50
261	Collagen a1 XIX chain	BAA23578	114354/8.66	DNA binding	20%	(Outside)	0	-0.74
263	Peroxisomal acyl-CoA oxidase	AAB62926	74660/8.74	Enzyme	16%	(Microbody)	0	-0.28
264	Immunoglobulin heavy chain	CAA27326	50073/6.41	Antibody	15%	(Cytoplasmic)	0	-0.20
265	Conserved helix-loop-helix ubiquitous kinase	AAC52589	84729/6.32	Enzyme	19%	cytoplasmic	0	-0.28
266[E]	Cytochrome c. somatic	P0009 [a]	11474/9.61	Transport	21%	Mitochondrial matrix (Necleus)	0	-0.78
267	MHC class H-2Dp	AAB62678	32053/5.57	Transport	17%	Integral to membrane (Microbody)	0	-0.71
268	Leucine zipper protein	CAA75400	56897/6.31	Receptor	21%	(Microbody)	0	-0.85
20 N	Leucine zipper protein	CAA75400	56897/6.31	Receptor	25%	(Cytoplasmic)	0	-0.85
3 N	BTG3	Z7200	28983/8.94	Regulate	25%	(ER)	0	-0.70

4 N	Nitrilase 1	AAC40185	35705/8.21	Enzyme	24%	(Cytoplasmic)	0	-0.16
11 N	Nitrilase 1	AAC40185	35705/8.21	Enzyme	17%	(Cytoplasmic)	0	-0.16
5 N	Betaine homocysteine methyl transferase	AAB87501	45021/8.01	Enzyme	25%	(Cytoplasmic)	0	-0.36
12 N	Betaine homocysteine methyl transferase	AAB87501	45021/8.01	Enzyme	37%	(Cytoplasmic)	0	-0.36
13 N	Betaine homocysteine methyl transferase	AAB87501	45021/8.01	Enzyme	28%	(Cytoplasmic)	0	-0.36
14 N	Betaine homocysteine methyl transferase	AAB87501	45021/8.01	Enzyme	15%	(Cytoplasmic)	0	-0.36
3-15	Betaine homocysteine methyl transferase	AAB87501	45021/8.01	Enzyme	16%	Cytoplasmic (Cytoplasmic)	0	-0.36
3-13[E]	Betaine--homocysteine S-methyltransferase	O35490 [a]	45448/8.01	Enzyme	23%	Cytoplasmic (Cytoplasmic)	0	-0.36
7 N	Putative CCR4 protein	AAB62717	36384/5.45	Unknown	16%	(Microbody)	0	-0.28

8 N	ADAM 4 protein precursor	AAA74922	51795/6.64	Enzyme	21%	Type I membrane protein (PM)	1	-0.25
9 N	NK 10 gene product	CAA56225	72423/8.91	Unknown	19%	（Microbody）	0	-0.98
10 N	TDAG8	AAB02188	39407/8.61	Receptor	15%	Integral membrane protein (PM)	7	0.206
15 N	MHCH-2Dr	AAA39605	40540/5.97	Transport	21%	Integral to plasma membrane (PM)	2	-0.43
17 N	tyrosinase	BAA00340	31121/5.22	Enzyme	21%	Type I membrane protein (Outside)	1	-0.25
18 N	Epsilon tyrosine phosphatase	AAC52281	80628/6.70	Enzyme	21%	Type I membrane protein (PM)	1	-0.35
19 N	Phosphatase 2A catalytic subunit isotype beta	CAA91559	35575/5.21	Enzyme	17%	(Cytoplasmic)	0	-0.44
21 N	X-linked inhibitor of apoptosis	AAB58376	55992/5.76	Enzyme	23%	(Microbody)	0	-0.59
22 N	Protein 4.1	AAA37123	78850/5.98	Cytoskeleton	23%	(Microbody)	0	-1.18

3-45	Protein 4.1	AAA37123	78850/5.98	Cytoskeleton	17%	(microbody)	0	-0.72
23 N	Tyrosine kinase	AAA40518	71574/6.75	Enzyme	23%	Cytoplasmic)	0	-0.44
3-42	Tyrosine kinase	AAA40518	71574/6.75	Enzyme	23%	(Cytoplasmic)	0	-0.44
24 N	Putative	AAA37550	53243/5.42	Unknown	31%	(MIS)	0	-0.60
3-16	Tyrosine kinase	AAA40518	71574/6.75	Enzyme	21%	Inner membrane (Cytoplasmic)	0	-0.44
25 N	MB2w3	CAA62323	28860/6.28	Receptor	22%	(PM)	2	0.024
28 N	Tyrosine-specific protein kinase	BAA05331	55594/6.30	Enzyme	24%	(MMS)	0	-0.15
29 N	17beta-hydroxysteroid dehydrogenase IV	CAA62015	79524/8.76	Enzyme	19%	microbody	0	-0.11
31 N	filensin	CAA73928	69909/5.78	Connecting	23%	(MMS)	0	-0.76
3-31	Filensin	CAA73928	69909/5.78	Connecting	23%	Membrane and cytoskeleton-associated	0	-0.76
32 N	Thyroid hormone receptor	CAA30575	46795/7.78	Receptor	23%	(Nucleus)	0	-0.44

3-1	Anticoagulant protein C	AAC33795	51818/5.95	Enzyme	28%	Plasma membrane (Outside)	0	-0.33
3-2	Interleukin-4 receptor precursor	AAA39300	26456/5.57	Receptor	22%	Type I membrane protein and secreted (Outside)	1	-0123
2-3	Interleukin 10 precursor	AAA39274	20641/8.45	Receptor	26%	Type I membrane protein	0	-0.28
2-4	Myosin heavy chain	AAA39790	11691/5.21	Cytoskeleton	50%	(Necleus)	0	-0.45
2-7	Cyclophilin	AAA37498	22713/9.48	Enzyme	25%	Integral membrane protein (outside)	1	-0.14
2-7[E]	Cyclophilin	AAA37498 [a]	22713/9.48	Enzyme	25%	Integral membrane protein (Bacteria cytoplasma)	1	-0.14
2-9	Collagen IV alpha subunit	AAA37342	25992/8.77	Cytoskeleton	37%	(Microbody)	0	-0.79
2-10	Inhibin beta-A subunit	CAA49325	47392/8.30	Enzyme	19%	(Outside)	0	-0.52
2-16	Monoclonal anti-DNA IgM heavy chain variable region	AAC26770	11900/9.04	Antibody	42%	(Microbody)	0	-0.66
3-14	Cyclic nucleotide	AAB03319	64471/5.67	Enzyme	23%	(Nucleus)	0	-0.30

	phosphodiesterase							
3-17	Meprin beta-subunit	AAA75234	79549/5.59	Enzyme	17%	Type I membrane protein (PM)	1	-0.33
3-22	MSG2 gamma salivary protein	AAB93512	16158/6.89	Detoxification	16%	Extracellular (Lysosome)	1	-0.37
3-25[E]	60 kDa heat shock protein	P19226	61088/5.91	Chaperone	27%	(Mitochondrial)	0	-0.08
3-28	Heat shock protein	AAA99485	96870/5.50	Chaperone	16%	(Nucleus)	0	-0.59
3-26	Plasminogen	AAA50168	90847/6.08	Enzyme	16%	Secreted (Outside)	0	-0.69
3-29[E]	ATP synthase D chain	Q9DCX2	18664/5.52	Enzyme	41%	(MMS)	0	-0.66
3-39	Unknown	AAA37266	46584/8.36	Unknown	19%	(Cytoplasmic)	0	-0.36
3-43	Immunoglobulin Heavy chain V-region	AAA38064	13299/8.50	Antibody	52%	(microbody)	0	-0.51
3-44	Phosphatidylcholine binding immunoglobulin heavy chain IgM variable region	AAB07419	11425/4.81	Antibody	67%	(microbody)	0	-0.39
3-46	Integrin beta3 subunit	AAB94086	86695/5.14	Receptor	20%	Type I membrane protein	3	-0.26

						(PM)		
3-49	Sp-alpha	AAB70571	38863/5.01	Regulation	19%	Secreted (PM)	3	0.718
3-50[E]	Prohibitin	P24142[a]	29859/5.57	Receptor	21%	(ERM)	0	0.009
3-51	Type II transforming growth factor-beta receptor	AAB30100	64576/6.02	Receptor	22%	Type I membrane protein (MIM)	2	-0.33
3-52	Myeloid upregulated protein	CAA04870	32098/8.23	Hematopoietic	22%	Integral membrane protein (PM)	8	0.718
3-53	Biliary glycoprotein	CAA53699	29984/9.22	Receptor	16%	Type I membrane protein (PM)	2	-0.00
3-56	TL antigen	CAA26859	40198/5.39	Antigen	17%	Type I membrane protein (PM)	1	-0.57
Lane-3	Similar to aminopeptidase N	XP_355626	120381/8.56	Enzyme	12%	Integral to membrane	6	0.144
Lane-3	Anpep protein	AAH17011	110721/5.62	Enzyme	25%	Integral to membrane	1	-0.25
Lane-3	Alanyl aminopeptidase	NP_032512	110022/5.62	Enzyme	23%	Integral to membrane	1	-0.27

Lane-5	ADP, ATP carrier protein	P51881	32931/9.74	Enzyme	15%	Integral membrane protein	6	0.019
Lane-5	Sodium channel 3*	AAA67107	49466/5.20	Channel	18%	Integral membrane protein	6	0.042
Lane-5	Unnamed protein product	BAB26162	30107/5.4	Unknown	23%	Integral to membrane	6	0.144
Lane-5	ZnT-1*	AAA79233	54716/6.20	Transport	16%	Integral membrane protein.	5	0.053
Lane-5	Dipeptidyl-peptidase IV α-chain	S23752	88093/5.96	Enzyme	11%	Integral to membrane	1	-0.32
Lane-5	Dipeptidylpeptidase 4	NP_034204	88065/5.96	Enzyme	11%	Integral to membrane	1	-0.32
Lane-11	DELTA-like 1*	CAA56865	78449/5.90	Antigen	20%	Type I membrane protein (PM)	2	-0.44
Lane-11	Expressed sequence AI788959	NP_705826	61351/7.96	Unknown	16%	Integral to membrane	2	-0.00
Lane-11	Ugt1a1 protein	AAH19434	57495/8.57	Enzyme	20%	Integral to membrane	1	-0.03
Lane-12	UDPglucuronosyltransferase 2 family, member 5	NP_033493	61386/7.94	Enzyme	33%	Unknown	2	-0.03
Lane-12	UDP glycosyltransferase 1 family polypeptide A13	AAP48600	61130/8.4	Enzyme	22%	Integral to membrane	2	0.036
Lane-12	UDP glycosyltransferase 1 family, polypeptide A6	NP_659545	61026/8.84	Enzyme	20%	Integral to membrane	2	-0.00

Lane-12	UDP glycosyltransferase 1 family polypeptide A7	AAP48596	61025/8.91	Enzyme	22%	Integral to membrane	2	0.016
Lane-12	UDP glycosyltransferase 1 family polypeptide A9	AAP48597	61018/8.91	Enzyme	14%	Integral to membrane	2	-0.01
Lane-12	Glucuronosyltransferase Ugt1.6	A55788	61017/8.91	Enzyme	20%	Integral to membrane	2	-0.00
Lane-12	Pheno 1 UDPglucuronosyltransferase	BAA13483	60992/8.92	Enzyme	14%	Integral to membrane	2	0.001
Lane-12	UDP glucuronosyltransferase 1 family, member 2	NP_038729	60987/8.3	Enzyme	14%	Integral to membrane	2	0.074
Lane-12	UDP glycosyltransferase 1 family polypeptide A2	AAP48594	60957/8.3	Enzyme	14%	Integral to membrane	2	0.075
Lane-12	UDP glycosyltransferase 1 family polypeptide A5	AAP48595	60686/8.33	Enzyme	18%	Integral to membrane	2	0.044
Lane-12	UDP glycosyltransferase 1 family polypeptide A10	AAP48598	60529/9.01	Enzyme	26%	Integral to membrane	2	-0.00
Lane-12	Unnamed protein product	BAC87656	60460/8.64	Unknown	30%	Unknown	2	0.042
Lane-12	UDP-glucuronosyltransferase 1-7 precursor	Q62452	59896/8.64	Enzyme	19%	Integral to membrane	2	0.046
Lane-12	bilirubin/phenol UDPglucuronosyltransferase	AAA40524	57569/8.57	Enzyme	20%	Integral to membrane	1	-0.02
Lane-13	Ugt2b5 protein	AAH28262	61404/7.94	Enzyme	33%	Unknown	2	-0.03

Lane-14	Testosterone 16-α-hydroxylase	AAA79023	57593/6.16	Enzyme	25%	Integral to membrane	1	-0.07
Lane-14	Cytochrome P450 2F2	P33267	56141/7.74	Enzyme	29%	Extracellular space	1	-0.13
Lane-14	Cytochrome P450, family2, subfamily f, polypeptide 2	NP_031843	56139/7.74	Enzyme	29%	Membrane	1	-0144
Lane-14	ADAM 4 protein precursor*	AAA74922	51795/6.64	Enzyme	18%	Type I membrane protein	1	-0.25
Lane-15	Class Ib MHC antigen Qa-2 precursor	AAB41657	37642/5.62	Antigen	28%	Integral to membrane	3	-0.35
Lane-15	H-2 class I histocompatibility antigen Q8 α-chain precursor	B24582	37330/5.52	Antigen	28%	Integral to membrane	3	-0.33
Lane-15	MHC H2-K antigen	AAA39652	41758/6.22	Antigen	31%	Integral to membrane	2	-0.37
Lane-15	H-2K (d) antigen	CAA25956	41750/6.02	Antigen	31%	Integral to membrane	2	-0.37
Lane-15	H-2 class I histocompatibility antigen, k-d alpha chain precursor (H-2K (d))	P01902	41749/6.16	Antigen	31%	Integral to membrane	2	-0.37
Lane-15	H2-K gene product	AAA80453	41603/6.02	Immune	25%	Integral to membrane	2	-0.37
Lane-15	H-2 class I histocompatibility antigen pH-2D-24	A21198	40148/7.7	Antigen	32%	Integral to membrane	2	-0.44
Lane-15	Similar to H-2 class I histocompatibility antigen, q8 α-chain precursor	XP_207059	35189/5.35	Antigen	26%	Integral to membrane	2	-0.45

	α-chain precursor							
Lane-15	Cytochrome P450 2D10	A30247	57554/6.16	Enzyme	25%	Integral to membrane	1	-0.08
Lane-15	MHC class I heavy chain maturation peptide H-2K (d)	AAR89506	39703/6.02	Immune	33%	Integral to membrane	1	-0.46
Lane-15	Major histocompatibility complex HLA I	1401243A	39581/6.09	Unknown	33%	Membrane	1	-0.46
Lane-15	Qa-2 cell surface antigen	BAA14174	21234/5.63	Antigen	28%	Membrane	1	-0.60
Lane-15	Major urinary protein*	CAA26953	20694/4.90	Antigen	20%	Secret	1	-0.32

The spots representing the identified proteins are indicated in Fig.3-6. Protein database accession numbers were obtained on NCBI database or SWISS-PROT (a is indicat Proteins identified by tandem MS (E is indicated) were marked with superscript, the others were identified by MALDI-TOF. Proteins identified by both MALDI ESI-Q-TOF were marked with italic numbers. In the columns pI and MW, the theoretical pI and molecular mass values are listed. In the column "Subcellular location", annotated subcellular location for the corresponding protein in the SWISS-PROT database, and the predicted location according to Http://psort.nibb.ac.jp/form.html are gi The "GRAVY" and "TMHMM" were listed (predicted through http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). ER, endoplasma reticulum; ERM, endoplasma reticu membrane; PM, plasma membrane; MMS, mitochondrial matrix space; MIM, mitochondrial inner membrane; MIS, mitochondrial inner space. "*" indicate identified 2DE-MS and 1DE-MS/MS. Lane-X represents the strip in 1DE.