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Contributed Paper

Comparative Membrane Extraction Methods for Identifying Membrane Proteome of SW900 Squamous Lung Cancer Cell Line

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ABSTRACT

Although several methods for extracting and handling membrane proteins for proteomics experiments have been reported, the direct comparison of different methods has never been clarified for the identifying membrane proteome in lung cancer cell lines. This study was purposed to find a protocol suitable for membrane protein extraction and to identify the membrane proteome in lung cancer. Three detergent-based extraction methods including sequential detergent extraction using Triton X-100 and digitonin, Mem-PER Eukaryotic membrane protein extraction kit and sucrose gradient ultracentrifugation were used to enrich the membrane proteins from SW900 squamous lung cancer cell line. The membrane protein profiles separated by SDS-PAGE and two-dimensional electrophoresis (2-DE) were compared. Mem-PER Eukaryotic membrane protein extraction kit was our preferable method which showed the highest number of protein spots, compared to the other methods, and the good resolution of membrane protein separation on the protein profile. The three membrane protein profiles from three extraction methods showed 73 matched protein spots, in which 15 membrane proteins were successfully identified by MALDI-TOF MS and MS/MS analyses and served as membrane proteome which played an important role in squamous lung cancer.

Keywords: membrane protein, detergent extraction, Mem-PER extraction kit, sucrose gradient ultracentrifugation, lung cancer cell line.

1. INTRODUCTION

The membrane is a thin bilayer of phospholipid which provides a physical boundary and cellular communication with their environment. It contains a variety of proteins, such as receptors, growth factors, neurotransmitters, pumps and channels, transporters and adhesion molecules [1-8]. These proteins play important roles in the regulation of cellular processes such as proliferation, cytosketetal remodeling, adhesion

and apoptosis in eukaryotic cells. Over two-thirds of all known protein targets for drugs are membrane proteins [9]. Consequently, the analysis of membrane protein is important for understanding of fundamental biological processes and disease biomarker discovery. Various methods have been developed to separate membrane proteins, such as biotin-directed affinity purification [10], aqueous polymer two-phase systems [11] and magnetic beads with immobilized antibodies [12]. Although these methods typically resulted in the high purity of membrane proteins, the low recovery efficiency and the requirement of specialized equipment or large samples are the disadvantages. The contamination of other organelles in membrane protein fractions is also a problem in extraction, such as from mitochondria, endoplasmic reticulum and cytosolic proteins. In general, detergent is the most commonly used reagent for membrane permeabilization and membrane protein isolation. This is because of its amphipathic property, consisting of a polar or charged head group and an extended hydrophobic hydrocarbon chain, and the ability to form micelles in aqueous solution [13]. The membrane protein complex could be purified in its native form using the detergent and also necessary to keep the complexes intact after solubilization and purification. This could be happened during column chromatography and preparative electrophoresis but at lower concentrations [14]. However, each type of detergents provides different yields of membrane proteins and the efficiency of membrane protein extraction method also depends on the sample source and component [15-17].

The squamous cell carcinoma lung cancer, also called epidermoid carcinoma, has been reported at about 30% of all cases of non-small-cell lung carcinomas [18]. This is currently the most frequent cause of death over the world [19]. The membrane protein of these cells needed to be studied as a challenge for the basic knowledge of several further possibilities for diagnosis, pathogenicity and therapeutic purposes. This study was focused on finding out a suitable protocol for membrane protein extraction and the identification of membrane proteome in the squamous cells of lung cancer. Three detergent-based extraction methods, including Triton X-100 and digitonin, Mem-PER Eukaryotic membrane protein extraction kit and sucrose gradient ultracentrifugation were evaluated. The membrane protein profiles were analyzed by SDS-PAGE and two-dimensional electrophoresis (2-DE). The membrane proteome were identified by MALDI-TOF MS and MS/MS and searched for category classification, consisting of cellular component, molecular function and biological processes.

2. MATERIALS AND METHODS

2.1 Cells and Chemical Reagents

Human squamous cell lung cancer cell line SW900 (ATCC#HTB-59) was purchased from ATCC (Rockville, MD, USA). Leibovitz' s L-15 Medium, phosphate-buffer saline (PBS), sodium bicarbonate, D(+)-glucose anhydrous, HEPES, piperazine-N,N'-bis(ethanesulfonic acid) (PIPES), Pefablock SC and aprotinin were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), trypsin-EDTA (10x), sodium pyruvate solution 100 mM (10x), antibiotic-antimycotic (100x) were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Sucrose, immobiline™ Dry strips length 18 cm; pH 3-10 linear, IPG buffer (pH 3-10 linear), mineral oil, low molecular weight (MW) SDS marker were purchased from GE Healthcare (Uppsala, Sweden). EDTA was purchased from Ameresco (Solon, OH, USA). Sodium chloride, magnesium chloride and Triton X-

100 were purchased from J.T. Baker (Phillipsburg, NJ, USA). Acrylamide/Bis-acrylamide (37.5:1) solution, Tris, CHAPS and glycine were purchased from BioShop (Burlington, ON, Canada). Dithioerythreitol (DTE) was purchased from AppliChem (Darmstadt, Germany). Iodoacetamide was purchased from Fluka (Buchs, Switzerland). SYPRO®Ruby gel stain was purchased from Molecular Probes (Eugene, OR, USA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA).

2.2 Cell Culture

SW900 cells were cultured in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine and 10% FBS in a humidified incubator with 5% CO_2 at 37°C. The cells were harvested by treatment with trypsin-EDTA and centrifugation at 125×*g* for 5 min, washed two times with PBS and stored at -80°C until use.

2.3 Sample Preparation

2.3.1 Whole Cell Protein Extraction

SW900 cell pellet (5×10^6 cells) was resuspended in a lysis buffer, containing 7 M Urea, 4% CHAPS and 2 M Thiourea, and broken with ultrasonication probe on ice. Then whole cell lysate was centrifuged at 1200 ×*g* to discard debris. The supernatant was collected and the protein concentration was determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin (BSA) as standard.

2.3.2 Mem-PER Eukaryotic Membrane Protein Extraction Kit

The membrane proteins were extracted with Mem-PER Eukaryotic membrane protein extraction reagents (Pierce Biotechnology, Inc) followed the instruction from the manufacturer and the isolated membrane proteins were submitted to methanol/chloroform precipitation. Briefly, 100 µl of isolated membrane protein fraction was added with cool methanol, chloroform and distilled water with 600, 150 and 450 µl, respectively. Then the mixture solution was incubated on ice for 5 min. After centrifugation at 8000 ×*g* for 10 min, the supernatant was removed. The insoluble material was resuspended in 300 µl of cool methanol and centrifuged again. The last step of methanol washing was repeated at least one time. The pellet was allowed to dry at room temperature about 2-3 min.

2.3.3 Sequential Detergent Extraction

The sequential detergent extraction procedure was modified from Ramsby and Makowski [20]. Briefly, SW900 cells were lysed in 300 µl of digitonin buffer pH 6.8 (10 mM PIPES, 0.015% w/v digitonin, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 10 µl/ml Pefabloc SC and 10 µl/ml aprotinin) and incubated on ice for 5 min with vortex every 1 min. After centrifugation at 1200 ×*g* for 5 min at 4°C, the pellet was resuspended in 150 µl Triton X-100 buffer (10 mM PIPES, pH 7.4, 0.5% v/v Triton X-100, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 10 µl/ml Pefabloc SC and 10 µl/ml of aprotinin) and incubated on ice for 30 min with vortex every 5 min. After centrifugation at 8000 ×*g* for 10 min at 4°C, the supernatant was removed and the insoluble material was submitted to methanol/chloroform precipitation as described above.

2.3.4 Sucrose Gradient Ultracentrifugation

Sucrose intensity gradient ultracentifugation was modified from Matousek *et al.*[21] Briefly, SW900 cell pellet was resuspended in homogenization buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA, 0.15 M NaCl and 1 mM protease inhibitor) and sonicated on ice with ultrasonicator probe

for 3 min. The cell lysated solution was overlaid with 20-40% sucrose gradient solution (20 mM Tris, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA and 0.15 M NaCl) in 10 ml centrifuge tube by using the Auto Densi-Flow® (Labconco Corp. Kansas, Missouri). Then, the gradient centrifuge tube was transferred into a Beckman SW41 rotor (Beckman Instruments Inc., Fullerton, CA, USA) and centrifuged at 200,000 ×*g* at 4°C for 22 h. The sucrose density gradient fractions were collected gently removing from surface with Auto Densi-Flow® by 0.5 ml in each fraction. The membrane protein fractions were collected and subsequently submitted to methanol/chloroform precipitation as described above.

2.4 Protein Analysis Methods

2.4.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The membrane proteins from each method were analyzed by 12.5% polyacrylamide (1.0 mm×10 well) with loading amount 10 µg of protein each well. The proteins were resolved in the SDS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS). The SDS-PAGE was pre-run at constant voltage of 60 V for 10 min and then increased to 120 V for 1.30 h.

2.4.2 Two Dimensional Electrophoresis (2-DE)

The membrane protein solution (250 µg protein) from each method was mixed with a lysis buffer, containing 7 M Urea, 4% CHAPS, 2 M Thiourea, 65 mM DTE and 0.5% IPG buffer pH 3-10, with a final volume of 350 µl. After sonication and centrifugation at 12000 ×*g* for 20 min, the protein solutions were applied onto IPG strips (18 cm, pH 3-10L; GE Healthcare). The first-dimensional isoelectric focusing (IEF) was performed on IPGphor IEF (GE Healthcare) under the following condition: 30 V, 14 h (rehydration); 100 V, 1 h; 250 V, 1 h; 500 V, 0.5 h; 1000 V, 0.5 h; 2000 V, 0.5 h; 4000 V, 0.5 h; 8000 V, 70 kVh. After IEF, the IPG strips were equilibrated with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2% w/v DTE and a trace of bromophenol blue) for 15 min, and then subsequently alkylated in the same equilibration buffer, but replacing DTE with 2.5% w/v iodoacetamide for 15 min. Thereafter, the IPG strips were placed onto 12.5% linear gradient polyacrylamide gel (18×18 cm) and covered with 0.5% agarose. The second-dimensional separation was performed in a Protean xi Multi-Cells (Bio-Rad) at 45 mA *per* gel at 15°C until the bromophenol blue dye front reached the bottom of the gel.

2.5 SYPRO® Ruby Staining

After SDS-PAGE or 2-DE, the gels were stained with SYPRO® Ruby (Molecular Probes), according to the protocol provided by the manufacturer. Briefly, the gels were incubated with a fixing solution, containing 50% methanol and 7% acetic acid with shaking for 1 h. The gels were incubated in SYPRO® Ruby gel stain solution overnight with gentle agitation. The gels were transferred to a clean container and soaked in a washing solution, containing 10% methanol and 7% acetic acid for 1 h. After rinsing with deionized water, the gel images were scanned using a Typhoon 9200 laser scanner (GE Healthcare) and exported to the image analysis software program, using ImageMaster™ 2D Platinum software version 5.0 (GE Healthcare).

2.6 In-Gel Digestion

Protein spots were manually excised from the gels and transferred to 500 µl siliconized Eppendorfs. The gel pieces were washed twice with 200 µl of 50% ACN/25 mM ammonium bicarbonate buffer, pH 8.5,

for 15 min each. The gel pieces were then washed with 200 µl of 100% ACN and dried using a Speed-Vacuum concentrator. Dried gel pieces were swollen in 20 µl of 25 mM ammonium bicarbonate containing 5 ng/µl sequencing grade modified trypsin. Gel pieces were then crushed with a siliconized blue stick and incubated at 37°C for at least 16 h. Peptides were subsequently extracted twice with 50 µl of 50% ACN/5% TFA, then the extracted solutions were combined and dried using a SpeedVac concentrator. The peptide pellets were resuspended in 10 µl of 0.1% TFA and the suspended solutions were purified using ZipTip C18 (Millipore, Billerica, MA, USA). Ten microliters of sample were drawn up and down in the ZipTip for 10 times and the ZipTip was wash with 10 µl of 0.1% formic acid by drawing up and expelling the washing solution for three times. The peptides were eluted with 5 µl of 75% ACN/ 0.1% formic acid.

2.7 MALDI-MS and MS/MS Analyses

The samples were premixed in a ratio of 1:1 with matrix solution (5 mg/µl CHCA in 50% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate) and spotted onto the 96-wells format MALDI sample stage. Data was directed acquisition on the Q-TOF Ultima™ MALDI instrument (M@LDI™; Micromass, Manchester, UK) which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each well, as many parent ions meeting the predefined criteria (any peak within the m/z 800-3000 range with intensity above 10 count ± include/exclude list) were selected from the most intense peak for CID MS/MS using argon as the collision gas and a mass dependent ± 5V rolling collision energy until the end of the probe pattern was reached.

The MASCOT (http://www.matrixscience.com) search engine was used for peptide mass fingerprinting (PMF) and MS/MS ions search, assuming that peptides were monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Only one missed trypsin cleavage was allowed, and peptide mass tolerances of 50 ppm was used for PMF and MS/MS ions search, respectively. The search was performed using the Swiss-Prot or NCBI human protein database. In addition, the identified proteins were also searched against protein PMF databases via the program Protein Prospector MS-FIT (http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm).

2.8 Bioinformatics

We used the combination of databases to gain information on protein name and symbol, category classification of cellular component, molecular function and biological function and references. The protein search programs used the sequential order of databases: Swiss-Prot/TrEMBL (http://us.expasy.org/sprot), NCBI (http://www.ncbi.nlm.nih.gov), GeneCards (http://www.genecards.org/index.shtml) and in-house Bulk Gene Search System for protein version (BGSS, http://servx8.sinica.edu.tw/bgss-cgi-bin/protein.pl).

3. RESULTS AND DISCUSSION

3.1 SDS-PAGE Analysis of SW900 Membrane Proteins

According to the most frequently used method for membrane protein preparation, the detergent extraction [15-17], we chose the three membrane protein extraction methods to extract the membrane proteins from SW900 cells. The extraction efficiency of each method was evaluated and compared to each other by proteomic analysis. In the sequential detergent extraction method, Triton X-100

and digitonin were used to fractionate membrane proteins in SW900 cells. This is by the reason of the nonionic property of the Triton X-100 which solubilizes membrane lipids and releases organelle components [20]. Digitonin is a steroidal compound believed to form complex with plasma membrane protein, resulting in membrane permeabilization and the rapid release of soluble cytosolic components leaving behind intact cell hosts and heavy organelles. So this method is a mild extraction method as compared to other methods.

Considering the method of Mem-PER Eukaryotic membrane protein extraction kit, in which the phase separation technique was applied to isolate hydrophobic membrane protein from hydrophilic proteins by Triton X-114 nonionic detergent. This detergent is not only solubilizes membrane proteins but also separates them from hydrophobic proteins *via* phase partitioning at physiological temperature [22,23]. Unlike traditional protocols involving phase partitioning with Triton X-114 or X-100, this kit does not require preparation of a membrane fraction as a prerequisite for protein solubilization. The membrane proteins were extracted directly with high efficiency from the crude cell lysates with a standard benchtop microcentrifuge within one hour.

In the sucrose gradient ultracentrifugation method, the sucrose density gradient coupled with ultracentrifugation were used to separate Triton X-100 insoluble floating fraction from detergent-solubilized proteins [21]. The advantage of this method is sucrose as medium that cheap, soluble, broad range of densities can be prepared that are required for separating most organelles. However, sucrose has very viscous and hyperosmotic at high concentration.

For sequential detergent extraction and Mem-PER Eukaryotic membrane protein extraction kit, ten million cells of cell pellet contained about 4.57 mg proteins were used and the protein concentrations of isolated membrane proteins were 257 and 350 μg, respectively. For sucrose gradient ultracentrifugation, 30×10^{10} cells of cell pellet contained about 11.90 mg proteins were used and the protein concentration of isolated membrane proteins was about 420 μg. The recovery yields of membrane proteins isolated from each method were 5.62%, 7.66% and 3.53%, respectively. After membrane protein extraction, the membrane proteins obtained from the three extraction methods were analyzed on SDS-PAGE and visualized by SYPRO® Ruby staining. Each extraction method gave slightly different membrane protein patterns, as compare to the whole cell lysate (Figure 1). The membrane proteins could be enriched in all methods, especially at a MW range of 25-70 kDa. The numbers of membrane protein bands obtained from Mem-PER Eukaryotic membrane protein extraction kit and sucrose gradient ultracentrifugation were higher than that obtained from sequential detergent extraction. It is very clear from the result that the membrane protein bands obtained from sucrose gradient ultracentrifugation exhibited higher intensity than that obtained from the other two methods.

3.2 2-DE Analysis of SW900 Membrane Proteins

In compare the details of the membrane protein patterns from the three extraction methods, the 2-DE was used to separate the membrane proteins from SW900 cells. Considering on the MW and p*I* values, each extraction method gave slightly different membrane protein patterns, as compared to the whole cell lysate (Figure 2). Using Image Master™ 2D Platinum software, the total numbers of protein spots on 2-D gel images from whole cell lysate, sequential detergent

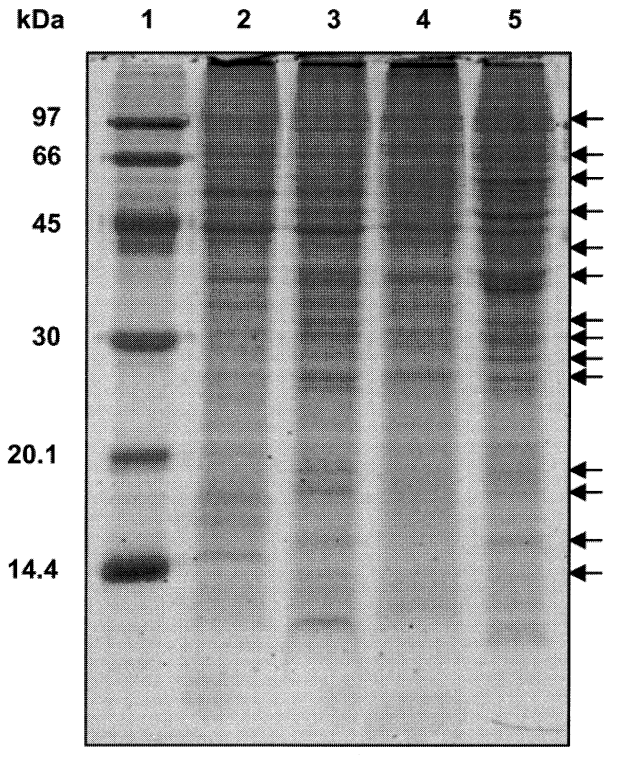

Figure 1. SDS-PAGE gel images of membrane proteins isolated from different extraction methods. The gel was stained with SYPRO® Ruby stain. Lanes: 1, protein marker; 2, whole cell lysate; 3, Mem-PER Eukaryotic membrane protein kit; 4, sequential detergent extraction; 5, sucrose gradient ultracentrifugation. Arrows represent the enriched membrane proteins.

extraction, Mem-PER Eukaryotic membrane protein extraction kit and sucrose gradient ultracentrifugation were 386, 495, 553 and 532 spots, respectively (Figure 3). This was clearly indicated that the membrane proteins from SW900 cells could be enriched by all the extraction methods. The total number of membrane protein spots obtained from sequential detergent extraction was less than that obtained from the other two methods and the 2-D gel profile showed some horizontal streaking in many areas. This might be because of the incomplete removal of the ionic impurity by the methanol/chloroform precipitation. Although we used non-ionic detergent instead of ionic detergent to extract membrane proteins, all buffer solutions still contained many ionic salts. In contrast, the membrane protein from Mem-PER Eukaryotic membrane protein extraction kit showed more clearly prominent profiles than that from the other two methods with the highest number of protein spots. This may be the consequences of the separation of the hydrophobic membrane proteins from hydrophilic proteins using the nonionic detergent Triton X-114 at physiological temperatures into detergent-rich and aqueous-rich phases [22,23]. However, the horizontal streaking was observed on the 2-DE gel due to the possibility of technically incomplete removal of the hydrophilic layer. On the other hand, many membrane protein spots on 2-DE gel from sucrose gradient ultracentrifugation were small and unclearly displayed, comparing to the other two extraction methods.

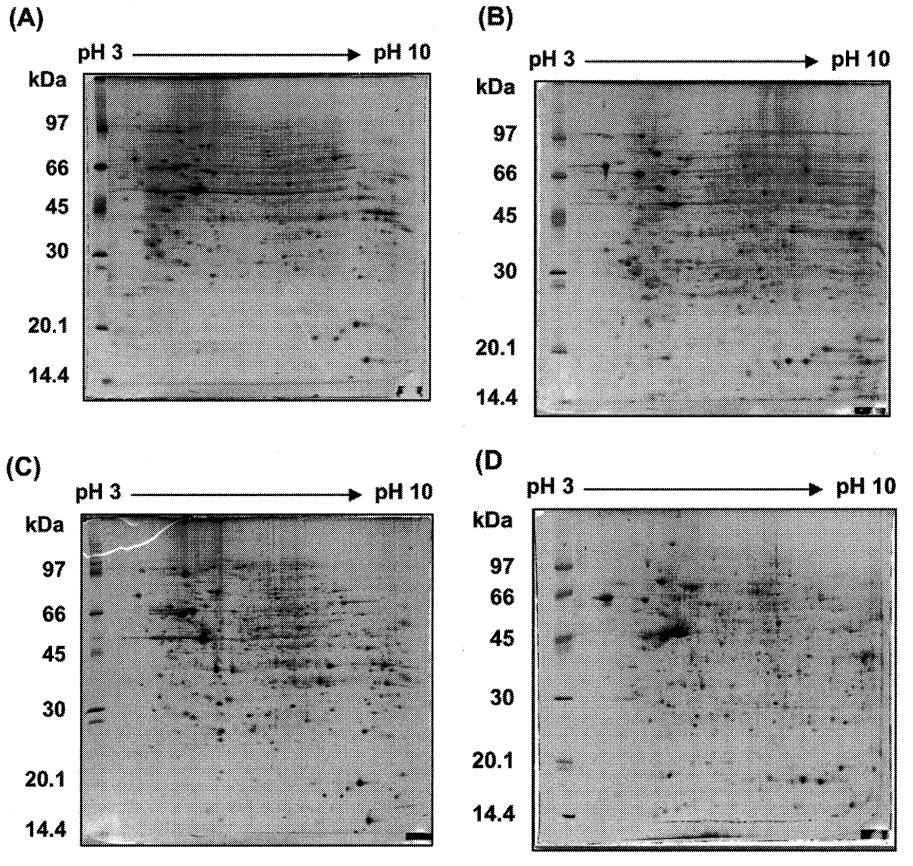

Figure 2. 2-DE gel images of whole cell lysate and membrane protein from squamous cell lung carcinoma cell line. Labels: (A) whole cell lysate; (B) sequential detergent extraction; (C) Mem-PER Eukaryotic membrane protein extraction kit; (D) sucrose gradient separation.

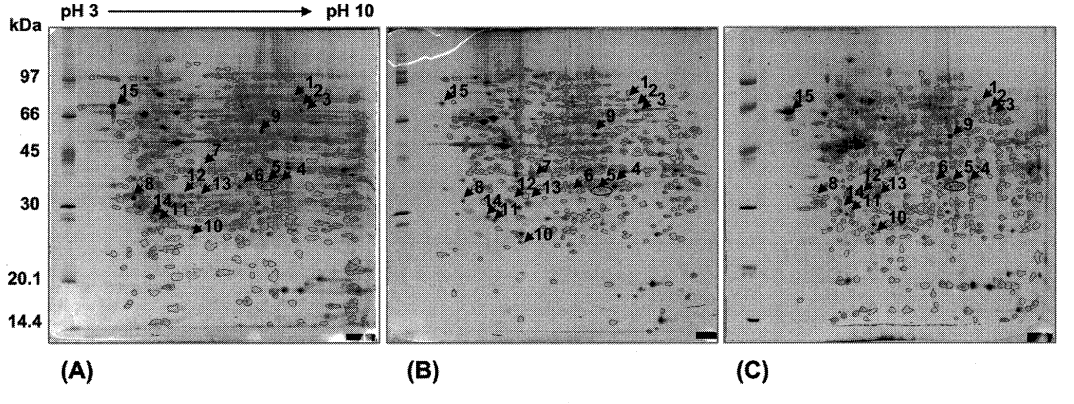

Figure 3. Quantitative detection of membrane protein spots from each extraction method by ImageMaster™ 2D Platinum software program. Labels: (A) sequential detergent extraction; (B) Mem-PER Eukaryotic membrane protein extraction kit; (C) sucrose gradient ultracentrifugation. The indicated numbers represent the membrane proteome that were identified by MALDI-TOF MS and MS/MS.

Although Triton X-100 extraction combined with sucrose gradient ultracentrifugation could separate detergent-resistant membrane proteins from detergent soluble material of non-raft regions [24-26], the obtained proteins have been found to be contaminated with elements of the endoplasmic reticulum, lysosomes, mitochondria and Golgi proteins [27,28].

In addition, the comparison of membrane protein spots between pair-wise extraction methods showed approximately 110 matched spots of sequential detergent extraction and Mem-PER Eukaryotic membrane extraction kit, 97 matched spots of sequential detergent extraction and sucrose gradient ultracentrifugation, and 144 matched spots of Mem-PER Eukaryotic membrane extraction kit and sucrose gradient ultracentrifugation (Figure 4). Considering the matched protein spots in each method, there were 134 (37.1%), 181 (48.7%) and 168 (46.2%) matched spots in sequential detergent extraction, Mem-PER Eukaryotic membrane extraction kit and sucrose gradient ultracentrifugation, respectively. It was indicated that Mem-PER Eukaryotic membrane extraction kit gave the membrane protein domain rather than the other two methods. Moreover, the comparison of three membrane protein profiles from three extraction methods showed 73 matched protein spots that were further identified by mass spectrometry.

3.3 Identification and Classification of Membrane Proteome

Among 73 matched protein spots, only 15 membrane proteins were successfully identified by MALDI-TOF MS and MS/MS (Table 1). The bioinformatics tools were used to search for protein annotation and category classifications, according to cellular component, molecular function and biological process

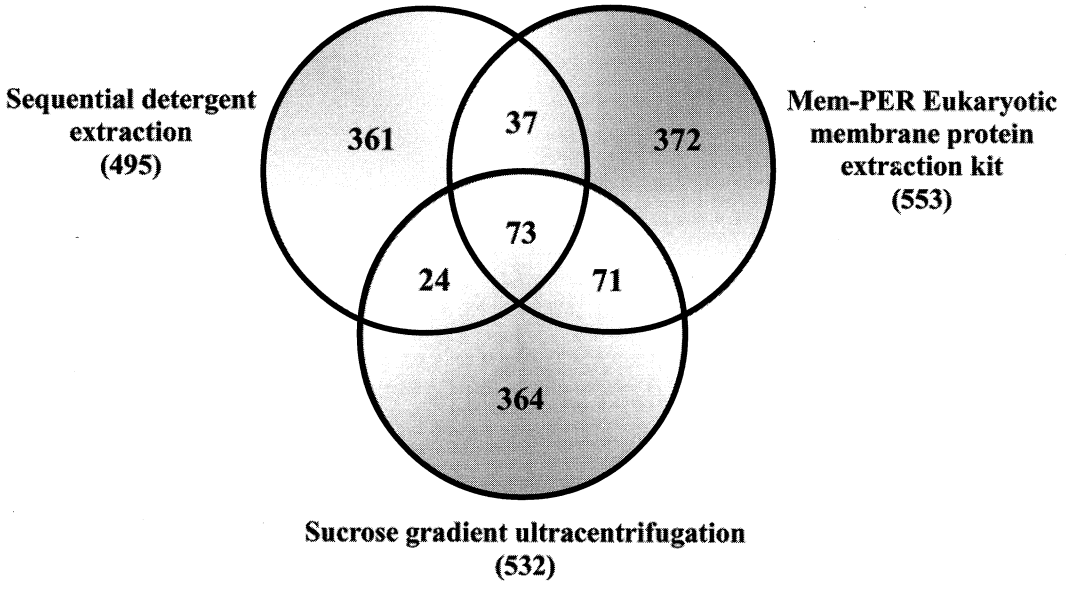

Figure 4. Pie chart of total membrane protein spots from three extraction methods: (A) sequential detergent extraction, (B) Mem-PER Eukaryotic membrane protein extraction kit, (C) sucrose gradient ultracentrifugation. The overlap pies represent the matching number of membrane proteins.

Table 1. Protein identification of membrane proteome in SW900 cells, which searched by MASCOT and MS-FIT software based on Swiss-Prot database.

Spot no.	Protein name	Accession no.	MS method	Apparent MW/p*I*	Theoretical MW/p*I*	Mowse score	Coverage	Match no.
1	Transketolase	P29401	MS	79770/8.0	67835/7.58	77	24	11
2	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	P49748	MS, MS/MS	78546/8.25	70345/8.92	142 ,48	36,2	18,1
3	Pyruvate kinase isozymes M1/M2	P14618	MS, MS/MS	74118/ 8.30	57900/7.96	62 ,64	28,3	11,1
4	Voltage-dependent anion-selective channel protein 2	P45880	MS	37230/7.65	31547/7.49	72	43	8
5	Electron transfer flavoprotein subunit alpha, mitochondrial precursor	P13804	MS, MS/MS	35890/7.5	35058/8.62	96,99	41,6	10,1
6	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor	Q13011	MS, MS/MS	35890/6.75	35793/8.16	153,103	51,4	17,1
7	L-lactate dehydrogenase B chain	P07195	MS	39640/5.85	36615/5.71	54	33	8
8	Peroxisomal biogenesis factor 19	P40855	MS	32950/4.20	32807/4.3	46.3	10.4	2
9	Alpha-enolase	P06733	MS, MS/MS	57710/7.35	47139/7.01	119,65	42,8	42,8
10	Peroxiredoxin-2	P32119	MS, MS/MS	25840/5.5	21878/5.66	54,41	30,6	6,1
11	Rho GDP-dissociation inhibitor 2	P52566	MS, MS/MS	28960/y4.95	22974/5.10	44,10	48,10	5,1
12	Deoxycytidine kinase	P27707	MS	32068/5.3	30519/5.1	26.9	10.0	2
13	Synaptosomal-associated protein 29	O95721	MS	32585/5.7	28971/5.6	63.1	19.4	5
14	Endoplasmin precursor	P14625	MS, MS/MS	100000/4.50	92411/4.76	93,44	17,1	14,1
15	Protein phosphatase 1 regulatory subunit 3F	Q6ZSY5	MS	77625/3.70	76102/4.4	147	7.1	3

(Table 2). Most of these proteins were distributed in the cell membrane (outer membrane and integral membrane), mitochondrion and cytoplasm, with 60% of all cellular components (Figure 5). They were also distributed in peroxisomal membrane, cytosol, nucleus, plasma membrane, cytoskeleton and microtubule, cytoplasmic membrane-bound vesicle, endoplasmic reticulum and others. This indicated that the membrane proteins can be transported from the outer cell membrane to the other organelles or regions *via* signal

Table 2. Category classification of identified proteins according to cellular component, molecular function and biological process.

Spot no.	Protein/Entry name	Accession no.	Category classification		
			Cellular component	Molecular function	Biological process
1	Transketolase [TKT]	P29401	-	Transketolase activity Calcium ion binding Protein binding Transferase activity	Metabolic process Regulation of growth
2	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor [ACADV]	P49748	Mitochondrion Mitochondrial inner membrane Membrane	Acyl-CoA dehydrogenase activity Long-chain-acyl-CoA dehydrogenase activity Oxidoreductase activity, acting on the CH-CH group of donors FAD binding	Electron transport Lipid metabolic process Fatty acid metabolic process Fatty acid beta-oxidation Metabolic process
3	Pyruvate kinase isozymes M1/M2 [KPYM]	P14618	Cytoplasm Mitochondrion Cytosol	Magnesium ion binding Pyruvate kinase activity Protein binding Transferase activity Potassium ion binding	Glycolysis
4	Voltage-dependent anion-selective channel protein 2 [VDAC2]	P45880	Mitochondrion Mitochondrial outer membrane Membrane Integral to membrane Outer membrane	Protein binding Voltage-gated ion-selective channel activity Porin activity Voltage-gated anion channel porin activity Voltage-gated anion channel activity	Anion transport
5	Electron transfer flavoprotein subunit alpha, mitochondrial precursor [ETFA]	P13804	Mitochondrion Mitochondrial matrix	Electron carrier activity FAD binding	Electron transport transport
6	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor [ECH1]	Q13011	Mitochondrion Peroxisome	Catalytic activity Enoyl-CoA hydratase activity Protein binding Isomerase activity	Generation of precursor metabolites and energy Lipid metabolic process Fatty acid metabolic process Fatty acid beta-oxidation metabolic process
7	L-lactate dehydrogenase B chain [LDHB]	P07195	Cytoplasm	L-lactate dehydrogenase activity Nucleotide binding GTP binding Oxidoreductase activity Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	Glycolysis Tricarboxylic acid cycle intermediate metabolic process Small GTPase mediated signal transduction Metabolic process Anaerobic glycolysis

Table 2. *(Continue)*

Spot no.	Protein/Entry name	Accession no.	Category classification		
			Cellular component	Molecular function	Biological process
8	Peroxisomal biogenesis factor 19 [PEX19]	P40855	Cytoplasm Peroxisome Peroxisomal membrane MembraneIntegral to membrane	Protein binding	Protein targeting to peroxisome
9	Alpha-enolase [ENOA]	P06733	phosphopyruvate hydratase complex Nucleus CytoplasmPlasma membrane Membrane	Magnesium ion binding Transcription factor activity Transcription corepressor activity Phosphopyruvate hydratase activity Protein binding	Negative regulation of transcription from RNA polymerase II promoter Glycolysis Transcription Negative regulation of cell growth
10	Peroxiredoxin-2 [PRDX2]	P32119	Cytoplasm	Thioredoxin peroxidase activity Antioxidant activity Oxidoreductase activity	Anti-apoptosis Response to oxidative stress Regulation of apoptosis
11	Rho GDP-dissociation inhibitor 2 [GDIR2]	P52566	Cytoplasm Cytoskeleton Cytoplasmic membrane-bound vesicle	Rho GDP-dissociation inhibitor activity GTPase activator activity Protein binding	Cell motility Immune response Negative regulation of cell adhesion Rho protein signal transduction Multicellular organismal development
12	Deoxycytidine kinase [DCK]	P27707	Nucleus	Nucleotide binding Deoxycytidine kinase activity ATP binding Kinase activity Transferase activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process Pyrimidine nucleotide metabolic process
13	Synaptosomal-associated protein 29 [SNP29]	O95721	Cytoplasm Plasma membrane Membrane Synaptosome Cell junction	SNAP receptor activity Protein binding	Exocytosis Vesicle targeting Membrane fusion Protein transport Vesicle-mediated transport
14	Endoplasmin precursor [ENPL]	P14625	Endoplasmic reticulum Endoplasmic reticulum lumen Endoplasmic reticulum membrane Microsome Cytosol	RNA binding Calcium ion binding ATP binding Transferase activity, transferring phosphorus-containing groups Virion binding	Response to hypoxia Protein folding Anti-apoptosis Protein transport Phosphorylation
15	Protein phosphatase 1 regulatory subunit 3F [PPR3F]	Q6ZSY5	Microtubule Membrane Integral to membrane	Leukotriene receptor activity GTP binding	Microtubule-based process G-protein coupled receptor protein signaling pathway

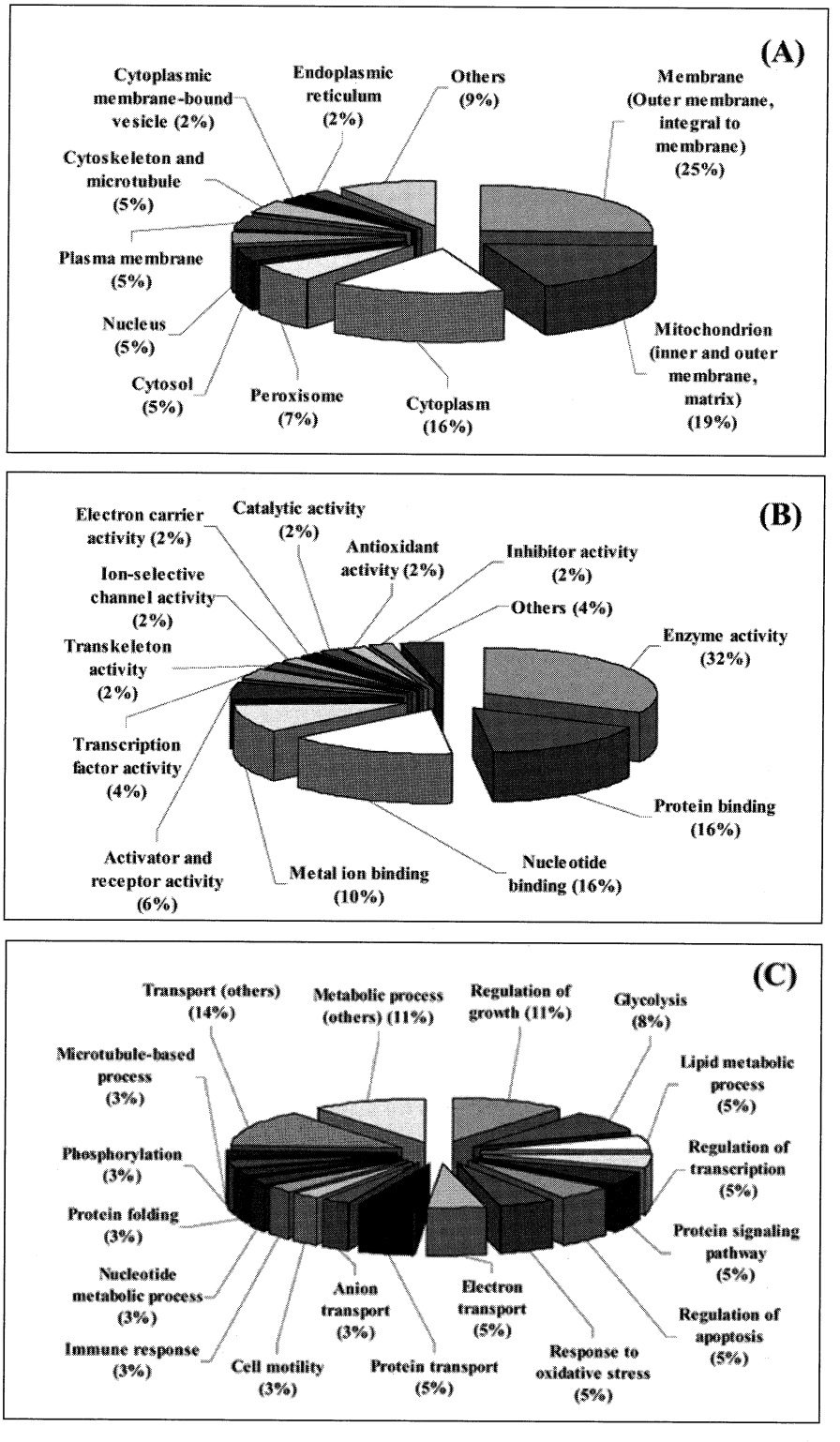

Figure 5. Category classification of identified membrane proteins according to cellular component (A), molecular function (B) and biological process (C) showing as percentage of the total protein.

transporters or targeting carriers [29,30]. These proteins possessed several molecular functions, such as enzyme activity, protein binding, nucleotide binding, metal ion binding, activator and receptor activity, transcription factor activity, transkeleton activity, ion-selective channel activity, electron carrier activity, catalytic activity, antioxidant activity and inhibitor activity. They were also found to play important roles in many biological processes, especially metabolic processes, regulation of growth, protein signaling pathway and regulation of apoptosis.

Based on our results, this indicated that all membrane extraction methods could enrich the membrane proteins in SW900 cells and showed nearly same efficiency. However, Mem-PER Eukaryotic membrane protein extraction kit gave the highest number of membrane protein spots, provided a good quality of membrane protein pattern and spent a shorter time (1 h) of membrane protein extraction, compared with the other two methods. Therefore, this method was the best for membrane protein extraction and suitable for further protein identification by proteomic analysis.

4. CONCLUSION

In summary, we applied three different membrane protein extraction methods to enrich the membrane proteins from SW900 cells and used proteomic analysis to examine the efficiency of membrane protein extraction methods, corresponding to their membrane protein profiles and quantification, and also to identify the membrane proteome. Among these methods, Mem-PER Eukaryotic membrane protein extraction kit was the effective method to enrich the membrane proteins in SW900 cells and gave a higher protein spot number and good quality of membrane protein profile, as compared with the other two methods. Most of the identified membrane proteins were distributed in several cellular components and have diverse molecular functions and biological processes. Therefore, this method could be used in further comparative study of the membrane proteome in each type of lung cancer cell lines, as well as compared to normal cell line, and to identify the unique biomarkers in lung cancer. However, the contamination in extracted membrane proteins is also the challenging issue in preparation of membrane protein due to the difficulties of contaminants removal.

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REFERENCES

[1] Exton J.H., Phosphoinositide phospholipases and G proteins in hormone action, *Annu. Rev. Physiol.*, 1994; **56**: 349-369.

[2] Patel T.B., Single transmembrane spanning heterotrimeric G protein-coupled receptors and their signaling cascades, *Pharmacol.Rev.*, 2004; **56**: 371-385.

[3] Grant S.G.N., Synapse signaling complexes and networks: machines underlying cognition, *BioEssays*, 2003; **24**: 1229-1235.

[4] Cobbold C. and Monaco A.P., Sivaprasadarao A., and Ponnambalam S., Aberrant trafficking of transmembrane proteins in human disease, *Trends Cell Biol.*, 2003; **13**: 639-647.

[5] Ballatori N., Transport of toxic metals by molecular mimicry, *Environ Health Perspect.*, 2002; **110**: 689-694.

[6] Jentsch T.J., Hübner C.A., and Fuhrmann J.C., Ion Channels: Function unraveled by dysfunction, *Nat. Cell Biol.*, 2004; **6**: 1039-1047.

[7] Hübner C.A., and Jentsch T.J., Ion channel diseases, *Hum. Mol. Genet.*, 2002; **11**: 2435-2445.

[8] Gumbiner B.M., Cell adhesion: The molecular basis of tissue architecture and morphogenesis, *Cell*, 1996; **84**: 345-357.

[9] Hipkins A.L., and Groom C.R., The druggable genome, *Nat. Rev. Drug discov.*, 2002; **1**: 727-730.

[10] Zhao Y., Zhang W., Kho Y., and Zhao Y., Proteomic analysis of integral plasma membrane proteins, *Anal. Chem.*, 2004; **76**: 1817-1823.

[11] Schindler J., and Nothwang H.G., Aqueous polymer two-phase systems: Effective tools for plasma membrane proteomics, *Proteomics*, 2006; **6**: 5409-5417.

[12] Lawson E.L., Clifton J.G., Huang F., Li X., Hixson D.C., and Josic D., Use of magnetic beads with immobilized monoclonal antibodies for isolation of highly pure plasma membranes, *Electrophoresis*, 2006; **27**: 2747-2758.

[13] Seddon A.M., Curnow P., and Booth P.J., Membrane proteins, lipids and detergents: not just a soap opera, *Biochim. Biophys. Acta*, 2004; **1666**: 105-117.

[14] Kashino Y., Separation methods in the analysis of protein membrane complexes, *J Chromatogr B Analyt Technol Biomed Life Sci.*, 2003; **797**: 191-216.

[15] Churchward M.A., Butt R.H., Lang J.C., Hsu K.K., and Coorssen J.R., Enhanced detergent extraction for analysis of membrane proteomes by two-dimensional gel electrophoresis, *Proteome Sci.*, 2005; **3**: 5.

[16] Helenius A., and Simons K., Solubilization of membranes by detergents, *Biochim. Biophys. Acta*, 1975; **415**: 29-79.

[17] Tanford C., and Reynolds J.A., Characterization of membrane proteins in detergent solutions, *Biochim. Biophys. Acta*, 1976; **457**: 133-170.

[18] American Thoracic Society , European Respiratory Society, Pretreatment evaluation of non-small-cell lung cancer, *Am J Respir Crit Care Med.*, 1997; **156**: 320-332.

[19] Jemal A., Riegel R., Ward E., Murray T., Xu J., and Thun M.J., Cancer statistics, 2007, *CA Cancer J. Clin.*, 2007; **57**: 47-66.

[20] Ramsby M.L., and Makowski G.S., Differential detergent fractionation of eukaryotic cells, *Methods Mol. Biol.*, 1998; **112**: 53-66.

[21] Matousek P., Hodny Z., Svadova I., and Svoboda P., Different methods of membrane domains isolation result in similar 2-D distribution patterns of membrane domain proteins, *Biochem. Cell Biol.*, 2003; **81**: 365-372.

[22] Qoronfleh M.W., Benton B., Ignacio R., and Kabord B., Selective enrichment of membrane proteins by partition phase separation for proteomic studies, *J. Biomed. Biotechnol.*, 2003; **4**: 249-255.

[23] Bordier C., Phase separation of integral membrane proteins in Triton X-114 solution, *J. Biol. Chem.*, 1981; **256**: 1604-1607.

[24] Babiychuk E.B., and Draeger A., Biochemical characterization of detergent-resistant membranes: a systematic approach, *Biochem. J.*, 2006; **397**: 407-416.

[25] Kim K.B., Lee J.S., and Ko Y.G., The isolation of detergent-resistant lipid rafts for two-dimensional electrophoresis, *Methods Mol. Biol.*, 2008; **424**: 413-422.

[26] Röper K., Corbeil D., and Buttner W.B., Retention of prominin in microvilli reveals distinct cholesterol-based lipid microdomains in the apical plasma membrane, *Nat. Cell Biol.*, 2000; **2**: 582-592.

[27] Fialka I, Pasquali C., Lottspeich F., Ahorn H., and Huber L.A., Subcellular fractionation of polarized epithelial cells and identification of organelle-specific proteins by two-dimensional gel electrophoresis, *Electrophoresis*, 1997; **18**: 2582-2590.

[28] Evans W.H., Fractionation of liver plasma membranes prepared by zonal centrifugation, *Biochem. J.*, 1970; **166**: 833-842.

[29] Chacinska A., and Rehling P., Moving proteins from the cytosol into mitochondria, *Biochem. Soc. Trans.*, 2004; **32**: 774-776.

[30] Neupert W., and Herrmann J.M., Translocation of proteins into mitochondria, *Annu. Rev. Biochem.*, 2007; **76**: 723-749.