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Application of displacement chromatography for the analysis of a lipid raft proteome

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ABSTRACT

Defining membrane proteomes is fundamental to understand the role of membrane proteins in biological processes and to find new targets for drug development. Usually multidimensional chromatography using step or gradient elution is applied for the separation of tryptic peptides of membrane proteins prior to their mass spectrometric analysis. Displacement chromatography (DC) offers several advantages that are helpful for proteome analysis. However, DC has so far been applied for proteomic investigations only in few cases. In this study we therefore applied DC in a multidimensional LC–MS approach for the separation and identification of membrane proteins located in cholesterol-enriched membrane microdomains (lipid rafts) obtained from rat kidney by density gradient centrifugation. The tryptic peptides were separated on a cation-exchange column in the displacement mode with spermine used as displacer. Fractions obtained from DC were analyzed using an HPLC-chip system coupled to an electrospray-ionization ion-trap mass spectrometer. This procedure yielded more than 400 highly significant peptide spectrum matches and led to the identification of more than 140 reliable protein hits within an established rat kidney lipid raft proteome. The majority of identified proteins were membrane proteins. In sum, our results demonstrate that DC is a suitable alternative to gradient elution separations for the identification of proteins via a multidimensional LC–MS approach.

1. Introduction

Membrane proteins play critical roles in many biological functions. They represent one-third of the proteins encoded by the human genome [1]. Plasma membrane proteins are responsible for key biological functions such as propagation of signaling cascades, intercellular communication, vesicle trafficking, protein translocation/integration and ion transport [2–4].

Lipid rafts are sphingolipid- and cholesterol-rich lipid domains different from other membrane surfaces, and are regions where many specific lipids and proteins, including signal transduction molecules, are associated with one another [5,6].

The analysis of membrane proteins poses a challenge since they are not easily soluble in polar solvents and often undergo aggregation and precipitation in aqueous buffers. Another problem is their general low abundance. Successful strategies for proteome analysis of membrane proteins must take into account both of these factors for sample preparation and analysis.

Conventional 2-DE is not well suited for the analysis of hydrophobic membrane proteins [7]. The higher the hydrophobicity of the protein, the lower the efficiency of separation by conventional 2-DE. This is among the above-mentioned characteristics of membrane proteins due to their poor solubility in the classical IEF buffers [8].

However, the bottom-up approach, also termed shotgun approach, has appeared as a powerful technique for membrane proteome identification. This approach starts with the enzymatic digestion of the proteins directly after the preparation of a membrane protein fraction. The resulting peptide mixture is extremely complex and therefore requires a 2- or more dimensional chromatographic separation prior to the mass spectrometric analysis. Multidimensional LC-separations are generally considered superior to 1-D-approaches for complex samples as the level of complexity is dramatically decreased whereby the mass spectrometric analysis is enhanced.

In 1943, Tiselius defined displacement chromatography besides two other chromatographic modes, elution and frontal chromatography [9]. Displacement chromatography is based on competitive

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binding of the components themselves and an additional molecule, the displacer. The column is firstly equilibrated with an eluent, termed "carrier". The carrier is used as a sample application buffer and has to support a high affinity of the sample components to the stationary phase. During sample loading, the sample components compete among themselves for the binding sites of the stationary phase. The component with the highest affinity to the stationary phase binds to the chromatographic material at the top of the column, displacing components with lower affinities from their binding sites. This process can be described as sample self-displacement [10]. Directly after sample loading, the displacer containing eluent is pumped onto the column. Usually the displacer is dissolved in the carrier. It is mandatory that the displacer has a very high affinity towards the stationary phase, ideally a higher affinity than any of the sample components. As soon as the displacer molecules adsorb the stationary phase they displace the sample component with the highest affinity that was bound on the top of the column. Each component will act as a displacing agent for neighbouring components of lower affinity towards the stationary phase. As a result, a system of contiguous zones with rectangular shape, termed "displacement train" will move down the column. The substances arrange each other in the order of their affinity to the stationary phase, the component with the lowest affinity to the stationary phase at the head of the displacement train and the most strongly retained directly in front of the displacer. Provided that the column is sufficiently long and the components are present in abundant amounts, each zone contains only one component in high purity [11]. The zones will keep their rectangular shape even if mass transfer resistances and slight kinetic or flow maldistribution are present. These effects are responsible for bandspreading in elution chromatography [12]. The displacement chromatography is finished if the displacer elutes in significant concentrations.

Displacement chromatography offers good recoveries due to its concentrating effect. Therefore, it is possible to analyze trace amounts of components of a complex mixture. The self-sharpening effect of the boundaries between the zones increases the efficacy of the separation.

For these reasons, displacement chromatography using a cation-exchange (CEX) material was applied as the first chromatographic step in a bottom-up proteomic approach including 2-dimensional liquid chromatography (LC) and LC–mass spectrometric (MS) analysis, investigating the composition of a lipid raft protein fraction.

2. Materials and methods

2.1. Preparation of kidney inner stripe lipid raft fractions

Male Wistar rats (300–350 g, $n = 3$) were bred in the local animal facility under standard conditions according to the German law for animal protection. For the isolation of lipid rafts, rats were anesthetized using isoflurane and killed by cervical dislocation. Kidneys were removed immediately and subjected to the protocol for lipid raft isolation as described previously [13]. Briefly, tissue of rat kidney inner stripe was minced in a mortar and homogenized in 250 mM sucrose, 10 mM triethanolamine (TEA) pH 7.5 and protease inhibitors (Complete™ Protease Inhibitor Cocktail, Roche Diagnostics). Tissue was subsequently lysed by ultrasonication (5× 5 s with 4 °C cooling intervals) to produce extracts. The homogenates were centrifuged at 300 × *g* (10 min, 4 °C) to remove nuclei and cell debris. The postnuclear supernatant was centrifuged at 120,000 × *g* (60 min, 4 °C) to concentrate the plasma membrane and cytoplasmic vesicular structures. Ice-cold Triton X-100 (final concentration of 1%, 60 min, 4 °C) was added and the detergent-insoluble fraction was resuspended in 40% sucrose in homogenization buffer. For the density gradient centrifugation, the sample was overlaid with 30% and 5% sucrose and centrifuged at 200,000 × *g* (16 h, 4 °C). From the gradient, 10 fractions were sequentially collected from the top of the gradient and analyzed by immunoblotting for the lipid raft marker protein anti-mouse-flotillin-1 and non-raft marker protein anti-mouse-transferrin receptor 1. Membrane raft proteins accumulated in the interphase of 5% and 20% sucrose.

2.2. Gel electrophoresis and immunoblotting

Preparation of membrane rafts was checked on 10% SDS polyacrylamide minigels. After electrophoretic transfer to nitrocellulose membranes, equity in protein loading and blotting was verified by membrane staining using 0.1% Ponceau red. Membranes were blocked with 5% skim milk in PBS and exposed to the specific antibodies for 90 min at room temperature, followed by HRP-conjugated secondary antibody (DAKO) for 45 min at room temperature (mouse monoclonal anti-flotillin-1, BD Transduction Laboratories, diluted 1:1000 in 5% skim milk in PBS; mouse monoclonal anti-human transferrin receptor 1, Alpha Diagnostic International, diluted 1:500 in 5% skim milk in PBS). Immunoreactive bands were detected by chemiluminescence using an enhanced chemiluminescence (ECL) kit and exposed to Amersham Hyperfilm ECL X-ray films (both from GE-Healthcare).

2.3. Membrane protein extraction and digestion

Raft fractions of all three animals were pooled and centrifuged at 100,000 × *g* for 60 min. Protein amount of the resulting pellet (BCA Protein Assay Kit, Pierce) was determined with a final concentration of 2 mg protein/ml. The pellet was resuspended in 25 mM NH_4HCO_3 pH 7.9 and washed 3 times by centrifugation at 100,000 × *g* for 60 min. Membrane proteins were extracted using 8 M urea according to Ruth et al. (2006). Briefly, the proteins were precipitated by the addition of 5 μl 8 M urea and then proteins were reduced by the addition of 200 mM DTT and incubation at RT for 60 min. Alkylation of proteins was carried out by the addition of 200 mM iodoacetamide for 60 min at RT. The solution was then buffered with 100 mM NH_4HCO_3 and immediately proteolyzed at 37 °C for 16 h with 1:200 trypsin (Sequencing Grade Modified Trypsin, Promega), dissolved in 100 mM $NaHCO_3$-buffer, pH 8.3. After the digestion, formic acid was added (final concentration of 0.2%) to the tryptic digest and the peptides were desalted by injecting (100 μl/min) the mixture onto a reversed-phase column (μRPC C2/C18 PC 3.2/3; GE-Healthcare; HPLC system: SMART, GE-Healthcare). After the salt was removed from the column, monitored by a conductivity detector, the peptides were eluted with an increasing gradient of acetonitrile (0–60% acetonitrile in 3 min).

2.4. Separation of the tryptic peptides by cation-exchange displacement chromatography

For the cation-exchange displacement chromatography a micro-preparative HPLC system (SMART; GE-Healthcare) was used. The desalted tryptic peptides were dissolved in 200 μl buffer A (5 mM phosphate buffer, pH 3, 30% acetonitrile) and injected onto the cation-exchange column (MiniS PC 3.2/3; GE-Healthcare). The displacer [14] (20 mM spermine, dissolved in buffer A) was pumped from the sample loop onto the column, directly after the tryptic peptides had been loaded to the column. The flow rate was 10 μl/min, fraction size was 5 μl.

2.5. Mass spectrometric identification of the lipid raft proteins

Liquid chromatography–tandem mass spectrometry analysis (LC–MS/MS) was performed using an ion-trap (IT) mass spectrometer (XCT, Agilent Technologies, Waldbronn), equipped with an

Agilent Chip Cube interface (Agilent Technologies, Waldbronn) and a silicon wafer "chip-column" (Agilent Technologies, Waldbronn) that integrates a C18 enrichment column, a C18 separation column and a nanospray emitter.

A 1100 capillary pump (Agilent Technologies, Waldbronn) working at 2 μl/min was used to pump HPLC-grade water with 0.2% (v/v) formic acid for sample pre-concentration (injection volume 2 μl) on the chip enrichment column (5 μm Zorbax 300 SB-C18 precolumn, 40 nl internal volume, Agilent Technologies). A 1100 nano-LC pump (Agilent Technologies) delivering, through flow splitting, 300 nl/min of a mixture of water/acetonitrile with 0.2% (v/v) formic acid was employed for the gradient separation. The tryptic peptide samples were eluted from the RP sorbent (5 μm Zorbax 300 SB-C18) of the chip separation column (75 μm × 50 μm cross-section, 43 mm length) using a linear gradient composed of the eluent A (water/0.2% formic acid) and the eluent B (acetonitrile). The gradient consisted of 3–15% of the eluent B over a 2 min and 15–45% of the eluent B over a 20 min program. The nanoelectrospray voltage was set at −2050 V. The IT-MS was scanning from 200 to 2000 *m/z*. Nitrogen at a flow rate of 4 l/min and heated to 325 °C was used as drying gas for spray desolvation. Precursor ion mass spectra were acquired at 0.5 s intervals in positive ion mode, with automated data-dependent MS/MS of the four most intense ions from each precursor MS scan. Doubly charged ions were preferably isolated and fragmented over single charged ions.

2.6. Bioinformatic analysis

Peaklists for MS/MS database search have been generated using Data Analysis Software for 6300 Series Ion Trap LC/MS version 3.4. Peptide identifications were performed using three different search engines. Mascot [15] version 2.1.03, OMSSA [16] version 2.1.1 and X!Tandem 2 [17] version 2007.07.01.1 were used to search the spectra against the rat subset of the Swiss-Prot database version 55.6, containing 7098 protein sequences [18]. The search parameters included variable carbamidomethylation on cysteine residues and oxidation on methionine residues. Only one missed trypsin cleavage was allowed during the searches. The precursor ion mass tolerance was set to 2.0 Da, the fragment ion mass tolerance was ±0.3 Da. Each of the searches was also performed against a reversed version of the database to assess the false positive rates [19,20]. The searches were performed using the search engine adapters of the OpenMS proteomics pipeline TOPP [21,22] version 1.2. The scores of the individual searches were converted to probabilities using the procedure described in [20]. Afterwards, a statistical model [23] was applied to infer probabilities of the proteins of the peptide hits. Only proteins with a reported probability of at least 0.99 and covered by at least two distinct peptides were kept for further analysis.

For interpreting and comparing the elution behaviour of both elution modes, the charge of each individual peptide was calculated based on the amino acid sequences determined by the database searches as described above and on the assumption, that each peptide is fully protonated.

3. Results and discussion

It was the aim of this study to test whether displacement chromatography is suited as a separating step in a multidimensional LC–MS bottom-up approach for the identification of membrane proteins. Therefore, proteins of rat kidney inner stripe lipid raft fractions were analyzed in a bottom-up approach. The experimental workflow of the proteome analysis is illustrated in Fig. 1. Lipid rafts from renal tissue were prepared using a standard protocol for lipid raft isolation including incubation of tissue homogenate on ice

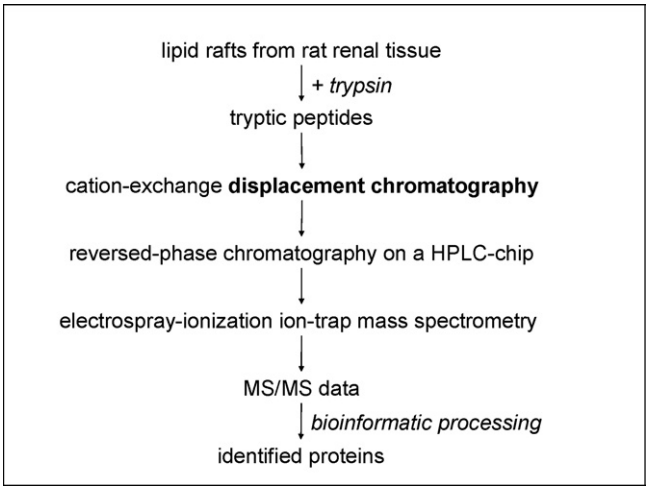

Fig. 1. Experimental workflow of the proteome analysis of a renal lipid raft fraction applying displacement chromatography.

with Triton X-100 and subsequent density gradient centrifugation. The obtained fractions were analyzed by SDS-PAGE and western blot using flotillin-1 as lipid raft marker and transferrin receptor 1 (transferrin R1) a typical protein which is not located in lipid rafts [24,25] (see Fig. 2). The separation of flotillin-1, a typical raft marker protein [26] from transferrin R1 in our floatation assay shows the successful separation of lipid rafts from non-raft domains.

Cultured cells usually are broken up by milder conditions, while kidney homogenates often are homogenized by a dounce homogenizer [27,28] or by suction through a syringe [25,29]. Kim et al. [32] also use the sonication step for the preparation of raft membranes

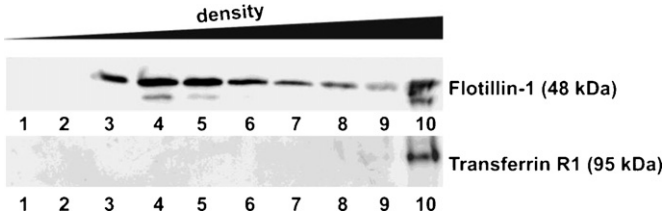

Fig. 2. Immunoblot analysis of a membrane raft assay with fraction 1 of lowest density and fraction 10 of highest density in a sucrose gradient. The low density raft fractions 3–6 are depicted by the raft marker protein flotillin-1 and can be distinguished from the non-raft fractions by the marker protein transferrin receptor 1, which is only present in fraction 10 of the density gradient.

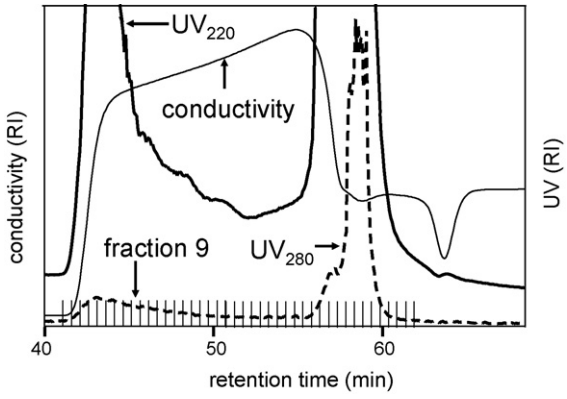

Fig. 3. Chromatogram of a cation-exchange displacement chromatography of the tryptic peptides of lipid raft proteins. Each fraction of the displacement chromatography was further analyzed by LC–MS. As an example the chromatogram of the LC–MS analysis of fraction 9 is shown in Fig. 4A.

and show immunoblotting of non-raft marker protein Clathrin which is lacking in raft membranes.

Nevertheless, the existence of mitochondrial proteins in lipid rafts has been mentioned in the previous works. Accordingly, Bae et al. conducting proteomics from rat liver lipid raft preparations [30], Kim et al. using 3T3 L1 adipocytes, HEK 293 cells or various mouse tissues [28,31,32], and also Yu et al. conducting a large scale proteomic analysis of isolated rat renal collecting ducts [33] found mitochondrial proteins, such as cytochrome *c* oxidase subunit Va, mitochondrial ATP synthase and voltage-dependent, anion-selective channels. However, there are no strong indications for the existence of mitochondrial rafts but some mitochondrial proteins have been shown to be localized in the plasma membrane [30] and therefore should also occur in lipid rafts.

Furthermore, a remarkable number of proteins of the endoplasmatic reticulum (ER) have been identified. The presence of rafts and raft proteins in the ER has been discussed in previous raft publications (e.g. in [5,34]). According to that, raft lipids and raft proteins are both synthesized in the endoplasmic reticulum before transport to the plasma membrane and the proteins are in a detergent-resistant state while residing there. For that reason these membrane parts should also accumulated in the lipid raft fraction.

Proteins of the raft fraction were enzymatically digested as the tryptic peptides are easier to handle than the membrane proteins they arise from. Nevertheless, the tryptic digest of such a complex protein mixture results in an even more complex mixture of peptides, deserving an excellent separation prior to their mass spectrometric analysis. Therefore, a 2-dimensional LC was applied. Displacement chromatography was used as the first separating step followed by a reversed-phase chromatography on an HPLC-chip system coupled to an online mass spectrometric analysis on an electrospray-ionization (ESI) ion-trap (IT) mass spectrometer. The resulting data were processed via bioinformatic tools and used for a database search for protein identification.

In Fig. 3, the result of the cation-exchange displacement chromatography is shown. Since the tryptic digest of the lipid raft proteins contains many hundreds of peptides, steps of a staircase-like chromatogram, which can be observed in the displacement separation of less heterogenic mixtures (e.g. in [35,36]), cannot be expected.

All 39 peptide-containing fractions of the displacement chromatography were analyzed using an HPLC-chip system. The

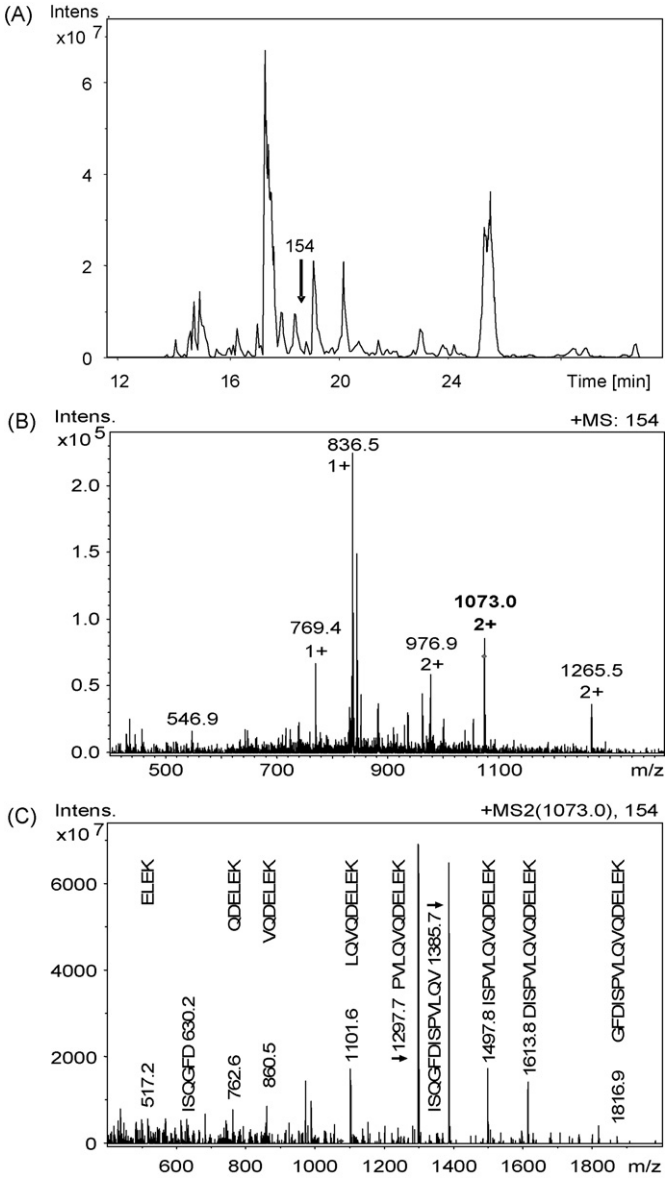

Fig. 4. (A) LC–MS chromatogram (total ion counts, arbitrary units) of the reversed-phase separation of fraction 9 from the cation-exchange chromatography. The chromatography was performed on an HPLC-chip. As an example the full scan spectrum measured at the retention time indicated by the arrow labelled with 154 (number of the full scan MS spectrum) is shown in (B). (B) Full scan MS spectrum of positive ions at the retention time as indicated by the arrow in (A). The doubly charged peptide ion (2+) underlying the signal at $m/z = 1073.0$ ($z = 2$) was selected for the MS/MS analysis (C). (C) MS/MS spectrum of the doubly charged peptide ion (2+) underlying the signal at $m/z = 1073.0$ ($z = 2$; indicated bold in the full scan spectrum in (B)). The identity of the individual peptide fragment ions is described by the one-letter amino acid code.

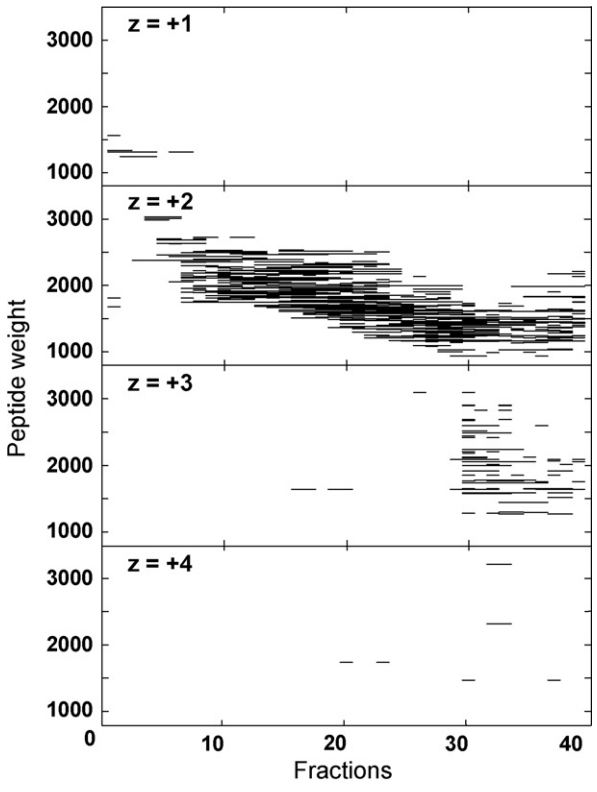

Fig. 5. Separation of peptides by displacement chromatography. Each line represents an individual peptide, characterized by its molecular mass (ordinate), and the size of the fraction (length of the line, abscissa). Peptides are separated by their predicted charge state (in the eluent buffer at pH 3); peptides with one positive charge are indicated by $z = +1$ (upper chromatogram); doubly charged peptides by $z = +2$ (second chromatogram), triply charged by $z = +3$ (third chromatogram) and peptides with four positive charges by $z = +4$ (fourth chromatogram).

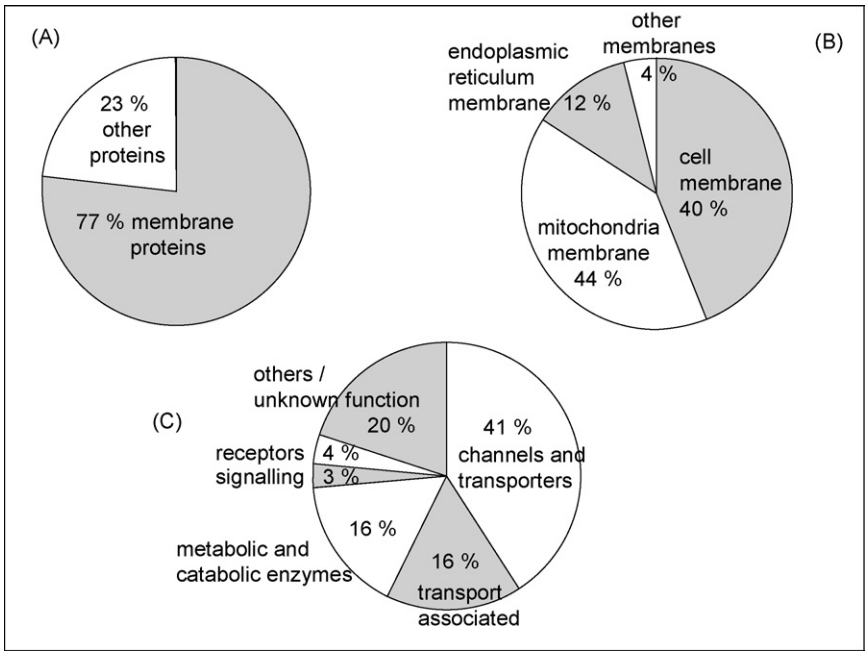

Fig. 6. (A) Characteristics of the identified proteins; 77% of the identified proteins were membrane proteins, and 23% were other proteins. The identified proteins are further segmented in (B) and (C). (B) Localization of the identified membrane proteins within the cell compartments. (C) Molecular functions of the identified membrane proteins.

HPLC-chip was equipped with a reversed-phase trapping and separating column, coupled to a nanoESI-IT mass spectrometer. A typical chromatogram of the LC–MS analysis is shown in Fig. 4A. The MS-data (Fig. 4B) of the LC–MS runs of the fractions obtained by gradient chromatography contained more than 11,500 measured MS/MS spectra (Fig. 4C). The information of these spectra was unified in one file and then used for the protein identification. Protein identification was performed by database search against a protein database using multiple search engines. This procedure yielded about 600 significant peptide spectrum matches (probability >0.95). More than 400 peptide spectrum matches of rat peptides were highly significant, leading to more than 140 reliable protein hits of rat thereof 108 were membrane proteins. The data have been evaluated in a very conservative way as the focus was not directed onto a high number of identified proteins but on the correctness of these identifications.

Fig. 5 presents a chromatogram of the cation-exchange chromatography displaying individual peptides as lines. This figure shows the retention behaviour of the tryptic peptides towards the cation-exchange chromatography material in the displacement mode. Since this kind of chromatogram is not very common, a detailed description of the steps applied for the generation of this chromatogram is given here. First, all peptides with score beyond 0.99 were selected. Second, the charge of each peptide was calculated assuming that negative groups such as carboxylic groups and basic groups of the peptides are completely protonated as the cation-exchange chromatography was performed at pH 3. Thus, a tryptic peptide with 1 basic amino acid has a positive charge of 2; one positive charge derives from the free amino terminus and the second positive charge from the additional amino group of the basic amino acid. Furthermore, the molecular mass of each peptide was calculated. In the chromatogram a horizontal line was drawn for each peptide at the corresponding molecular mass. The length of this line corresponds to the number of fractions in which this peptide was detected.

The peptides in the displacement chromatogram are well distributed in the separation space. Responsible for this phenomenon are the secondary interactions between the stationary phase and the analytes, which are much more pronounced in displacement than in gradient chromatography because the driving force in displacement chromatography is the competition of the sample components for the stationary phase [37,38].

A majority of the identified rat proteins (77%, see Fig. 6A) belong to the group of membrane proteins. This distribution shows the capability of the bottom-up approach in combination with displacement chromatography for the identification of membrane proteins. Many of them are in agreement with the current knowledge about lipid rafts, hence grounding the basis for new experiments to investigate the functions of lipid rafts.

Further exploration of the localization and molecular function revealed that 40% of the identified membrane proteins are localized in the plasma membrane (Fig. 6B and C). This result is consistent with the previous results of Bae et al. identifying 32% [30] or Foster et al. [5] identifying 34% plasma membrane proteins.

The results clearly demonstrate that displacement chromatography is a suitable tool for the separation of peptides and the subsequent identification of proteins. Therefore, it is an alternative to gradient elution chromatography as the first separating step in multidimensional chromatography following the shotgun approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.11.035.

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