EVALUATION OF FOUR DIFFERENT STRATEGIES TO CHARACTERIZE PLASMA MEMBRANE PROTEINS FROM BANANA ROOTS

Avaliação de quatro estratégias diferentes para caracterizar proteínas da membrana plasmática de raízes de banana

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ABSTRACT

Plasma membrane proteins constitute a very important class of proteins. They are involved in the transmission of external signals to the interior of the cell and selective transport of water, nutrients and ions across the plasma membrane. However, the study of plasma membrane proteins is challenging because of their poor solubility in aqueous media and low relative abundance. In this work, we evaluated four different strategies for the characterization of plasma membrane proteins from banana roots: (i) the aqueous-polymer two-phase system technique (ATPS) coupled to gelelectrophoresis (gel-based), and (ii) ATPS coupled to LC-MS/MS (gel free), (iii) a microsomal fraction and (iv) a full proteome, both coupled to LC-MS/MS. Our results show that the gel-based strategy is useful for protein visualization but has major limitations in terms of time reproducibility and efficiency. From the gel-free strategies, the microsomal-based strategy allowed the highest number of plasma membrane proteins to be identified, followed by the full proteome strategy and by the ATPS based strategy. The high yield of plasma membrane proteins provided by the microsomal fraction can be explained by the enrichment of membrane proteins in this fraction and the high throughput of the gel-free approach combined with the usage of a fast high-resolution mass spectrometer for the identification of proteins.

Index terms: Aqueous-polymer two-phase system technique, microsomal and full proteome fractions, protein identification.

RESUMO

Proteínas da membrana plasmática constituem uma importante classe de proteínas. Elas estão envolvidas na transmissão de sinais externos para o interior da célula e no transporte seletivo de nutrientes/ions, através da membrana plasmática. Porém, o estudo dessas proteinas é difícil, porque elas são poucoabundantes e apresentam baixa solubilidade em tampões aquosos. Neste trabalho, nós avaliamos quatro estratégias diferentes para extrair proteínas da membrana plasmática de raízes de banana: (i) a técnica de sistema aquoso de duas fases, constituída porpolímeros(ATPS) combinada com eletroforese em gel e (ii) ATPS sem gel usandoLC-MS/MS, (iii) uma fração microssomal e (iv) uma fração, contendo o proteoma total celular, ambas as frações avaliadas via LC-MS/MS. Nossos resultados mostram que a estratégia baseada em eletroforese em gel é útil para a visualização de proteínas, mas apresenta limitações em termos de reproducibilidade e eficiência. Dentre as estratégias sem o uso de gel, a fração microssomal permitiu a identificação do maior número de proteínas de membrana plasmática, seguida pela fração de proteoma total e pela técnica de sistema aquoso de duasfases. O alto rendimento de proteínas de membrana plasmática proporcionado pela fração microssomal pode ser explicado pelo enriquecimento de proteínas de membrana nessa fração e pela eficiência do espectrômetro de massa na identificação de proteínas.

Termos para indexação: Sistema aquoso de duas fases, frações microssomal e de proteoma total, identificação de proteínas.

INTRODUCTION

Plants are constantly exposed to environmental changes. The plasma membrane constitutes a selective barrier between the cell and the external environment. At the plasma membrane, proteins are located that regulate the traffic/transport of water and nutrients/ions in and out of the cells. Others are also involved in the transmission of extracellular signals and thus play a crucial role in cell signaling. Therefore, the identification and characterization of plasma membrane proteins are of utmost importance for understanding at the molecular level plant responses towards external factors. Roots are the first organ to sense the lack of soil water and to adapt for a better water uptake and retention.

The plasma membrane proteome constitutes only 1.5-6% of the total cell, which results in a relatively low

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Received in may 29, 2014 and approved in july 30, 2014

abundance of plasma membrane proteins compared to soluble/cytosolic proteins (Nilsson, et al., 2010). Furthermore, integral plasma membrane proteins exhibit a very poor solubility in an aqueous buffer due to the presence of hydrophobic transmembrane domains (TMD). This leads to protein aggregation and precipitation affecting protein isolation efficiency and enzymatic digestion for protein identification (Speers; Wu, 2007) making the characterization of these proteins challenging. Therefore the characterization of plasma membrane proteins requires an efficient protocol to isolate a sufficient quantity of plasma membrane proteinswith high purity.

Several techniques were developed for the isolation of plasma membranes such as sucrose gradient centrifugation (Valotet al., 2005), cationic colloidal silica (Chaney; Jacobson, 1983), biotinylation (Elia, 2008), glycosylation (Ghoshet al*.,* 2004) and affinity purification.

One of the most used techniques for the isolation of plasma membrane proteins is the aqueous-polymer two-phase partitioning first described by Larsson (1985). The development of this technique was based on the observation that two phases are formed when two structurally different water-soluble polymers are mixed above a certain concentration. PEG and dextran are the most used polymers, being the upper phase rich in PEG and the lower phase rich in dextran (Schindler; Nothwang, 2006). In addition to these polymers, also sucrose, buffers and salts are added to the aqueous two-phase system to create a suitable tonicity, pH and ion strength. Once the two phases are settled, a microsomal fraction, which consists of membrane vesicles from different origin, is added to the aqueous-polymer two-phase system (Larsson, 1985). The membrane vesicles are formed after cell disruption by an osmotic shock, ultrasonic vibration, or blending and pieces of plasma membranes and endomembranes are resealed into vesicles (Albertset al.,2002).

The membrane vesicles added to an aqueous-polymer two-phase system will partition between the upper phase, interphase and lower phase based on their affinity to the hydrophobic PEG upper phase. The affinity to the PEG phase is determined by the lipid composition and distribution across the membrane bilayer. The plasma membranes have the highest affinity for the hydrophobic PEG phase, therefore they will preferentially partition into the upper phase followed by the Golgi apparatus, lysosomes, endoplasmatic reticulum and mitochondria (Rito-Palomares, 2004) (Schindler; Nothwang, 2006). However, knowledge of the mechanism of protein partitioning into an aqueous-polymer two-phase system is still limited.

The lipid composition of the plasma membranes distinguishes from the lipid composition of the endomembranes especially by its enrichment in sphingolipids, phosphatidylserine and cholesterol. Furthermore, the plasma membranes possess a clear asymmetric arrangement, with the exoplasmic phase rich in glycosphingolipids and phosphatidylcholine and the cytoplasmic face rich in phosphatidylserine and phosphatidylethanolamine (Zachowski, 1993). This may be one of the reasons why the plasma membranes present the highest affinity for the hydrophobic PEG upper phase.

To obtain a high enrichment of the plasma membranes in the upper phase, and the endomembranes at the interphase and lower phase, the polymer and salt concentrations should be composed in a systematic way. Larsson (1985) reported for membrane vesicles extracted from wheat roots that an increase in the polymer concentration from 5.5% to 7.7% (w/w) and potassium chloride (KCl) from zero to 5 mM decreased the amount of endomembranes into the upper phase while the concentration of the plasma membranes was maintained high. However, the surface membrane properties may vary between species and tissues, therefore, this technique needs to be optimized for every species and tissue (Schindler; Nothwang, 2006).

Aqueous-two-phase systems have a wide range of applications. It goes from purification of pharmaceutics (Harris et al., 2007) and therapeutic proteins (Asenjoet al., 2012), to isolation of proteins from bacteria (Lutwycheet al*.,*1995) animals (Gierowet al., 1986; Boland et al., 1991) and plants. In plants, the aqueous two-phase system has been mostly used for protein isolation from Arabidopsis (DeWitt et al., 1996; Borneret al., 2003). However it has been used for isolating plasma membrane proteins from oat (Widell; Lundborg; Larsson 1982), tobacco (Platis; Nikolaos; Labro, 2006), orchidgrass (Yoshida; Uemura,1984), sweet potato (Srinivas; Rashmi; Raghavarao,1999) and banana leaves (Vertommenet al., 2011).

This is the first study applying the aqueous-polymer two-phase system technique to isolate plasma membrane proteins from banana roots. We tested four different polymer concentrations to assess the enrichment of plasma membrane proteins in the upper phase.

We evaluated four different strategies for the identification of plasma membrane proteins from banana roots. We will discuss the advantages and disadvantages of each strategy.

MATERIAL AND METHODS

Extraction of the full proteome

Roots (0,3g) of the banana cultivar Cachaco (ITC0643) cultivated in a hydroponic nutrient solution consisted of 350 mM KNO_3, 69 mM K_2SO_4, 71 mM $MgSO_4.7H_2O$, 89 mM $MgCl_2.6H_2O$, 3.68 mM H_3BO_3, 3.19 mM $MnSO_4.H_2O$, 0.186 mM $ZnSO_4.7H_2O$, 0.128 mM $CuSO_4.5H_2O$, 0.031 mM $NaMo_4.2H_2O$, 142.7 mM KH_2PO_4, 255.42 mM NaH_2PO_4, 196.28 mM $Ca(NO_3)_2.4H_2O$, 71.42 mM $CaCl_2.2H_2O$, sequestrene 12 gL^{-1}, pH 6.0 were crushed in liquid nitrogen using a mortar and pestle. The grounded tissue was mixed with 750 µl buffer extraction containing 5 mM EDTA, 100 mM KCl, 1% dithiothreitol (DTT), 30% sucrose, one mini tablet of protease inhibitor (Roche) and 100 mM Tris-HCl pH 8.3, and vortexed for 30 sec. Subsequently, 750 µl buffered phenol containing 1% bromophenol blue and 50mMtris-base were added and the sample was vortexed for 10 min at 4° C. The sample was centrifuged for 10 min at 12,000 rpm at 4° C and the phenolic phase was collected. An equal volume of the extraction buffer was added to the collected phenolic phase. The sample was then centrifuged for 5 min at 12,000 rpm at 4°C and the phenolic phase was re-extracted. The volume extracted from the phenolic phase was mixed for protein precipitation overnight at -20° C with 5 volumes of methanol solution containing 100 mM ammonium acetate. The sample was centrifuged for 60 min at 13,000 rpm at 4° C, the supernatant was removed and the pellet rinsed with 2 ml of cold acetone containing 0.2% DTT. The sample was incubated into the rinsing solution for 1 hour at -20° C followed by a centrifugation step of 30 min, at 13,000 rpm at 4° C. The supernatant was removed and the pellet was dried under the hood. The pellet was resuspended in 50 µl of lysis buffer containing 8 M urea, 5 mM DDT, 30 mM Tris and the sample was quantified using a 2-D Quant Kit (GE Healthcare). Twenty microgram proteins were treated with 0.02 M DTT for 15 min, followed by 30 min darkness incubation in the presence of 0.05 M iodoacetamide (IAA) and diluted in 3 volumes of ammonium bicarbonate (ABC) 0.05M. The protein sample was digested with 0.2 µg trypsin overnight at 37° C. Finally the sample was acidified in 0.1% of trifluoroacetic acid (TFA). The next steps regarding protein identification are reported in the Sections "Salt removal" and "Mass spectrometry analysis".

Isolation of membranes vesicles

Roots (200 g) of the banana cultivar Cachaco (greenhouse plants, ITC0643) were crushed using a cold kitchen coffee blender and homogenized in 600 mL ice cold homogenization buffer pH 7.0 containing: 50 mM MOPS, 5 mM EDTA, 0.33 M sucrose, complete EDTA-free protease inhibitor cocktail (Roche), 1% DTT (dithiothreitol) and 5 µM ascorbate. Sample heating was prevented by applying short blending strokes. The homogenate was filtered through 4 layers cloth filter. The cell debris, cell wall and nuclei were removed by centrifugation at 10,000 g for 10 min at 4°C. The supernatant (= total fraction) was submitted to ultracentrifugation at 100,000 g for 1 hour at 4°C to separate cellular membranes from soluble cellular components. After ultracentrifugation, a microsomal pellet containing plasma membranes and endomembrane vesicles was obtained. During the centrifugation steps one milliliter aliquot was collected from the total and soluble fractions for protein precipitation and separation in one dimension SDS-PAGE gel (Figure 1).

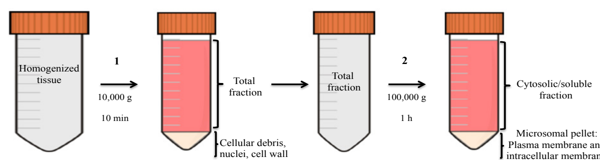

Figure 1 – Centrifugation steps used to obtain the microsomal pellet: 1) Homogenized Cachaco root tissue was centrifuged at 10,000 g for 10 min to precipitate cellular debris, nuclei and cell wall; 2) the total fraction (supernatant) was subsequently centrifuged at 100,000 g for 1 h to separate cellular soluble components from cellular membranes.

Aqueous-polymer two-phase partitioning

The microsomal pellets obtained from 200 g crushed roots were pooled and suspended in 40 mL buffer containing 0.33 M sucrose, 0.1M EDTA, 1% DTT, protease inhibitor cocktail and 5 mM potassium phosphate buffered at pH 7.8. After resuspension of the microsomal fraction, an aliquot of 200 µL was collected for protein extraction, precipitation and separation in one dimension SDS-PAGE gel. Four aliquots, each containing nine milliliters of the microsomal fraction were subsequently applied into four aqueous-polymer two-phase partitioning containing four different polymer concentrations: 6.2%, 6.4%, 6.8% and 7.2% (w/w) PEG 3350, and 6.2%, 6.4%, 6.8% and 7.2% (w/w) dextran T500 (w/w); and 11% (w/w) sucrose, 5 mM potassium phosphate (pH 7.8), 5 mM KCl and milli-Q water until a final weight of 27 g per system. Each aqueous two-phase system containing the microsomal

fraction was shaken followed by centrifugation at 1,800 g for 5 min at 4° C to enhance phase separation. The upper phase was re-extracted three times. The upper phase and the lower phase were diluted two times in resuspension buffer containing 0.33M sucrose and 5mMpotassium phosphate buffer pH 7.8, before centrifugation at 100,000 g for 1 hour at 4° C. Non membrane bound proteins were removed by resuspending the obtained pellets with 2 mL Brij58 buffered at pH 4.0 (150 mM KCl, 1mM EDTA, 0.01%. Brij58, 25 mM sodium acetate). Next, we applied a new ultracentrifugation round at 150,000 g for 1hour at 4° C. Then the pellets were resuspended and the proteins extracted from the membrane in SDS buffer 4% (100 mM Tris/ HCl pH 6.8, 4% SDS, 1 % DTT) and stored at -80° C.

Protein precipitation

Two hundred microliters of total, soluble and microsomal fractions, and upper and lower phases were diluted into 1.8 ml of cold acetone solution containing 10% of TCA (trichloroacetic acid) and 0.2% (DTT) for protein precipitation overnight at -20° C. Subsequently, the samples were centrifuged at 13,000 rpm for 30 min at 4° C. The supernatants were discarded and the pellets were washed with 2 ml of cold acetone containing 0.2% DTT and centrifuged again at 13,000 rpm for 30 min at 4° C. The supernatants were discarded and the pellets were dried under the hood. The proteins were then solubilized in SDS 4% buffer containing 100 mM HCl pH 7.6 and 0.1 M DTT. The protein concentration was determined using the micro-Bradford protocol (Zuo, Lundahl, 2000).

Protein separation in one dimension SDS-PAGE gel

An equal amount of the total, soluble and microsomal fractions, and upper and lower phases were loaded into a precast NuPAGE® Novex® Bis-Tris Mini gel (Invitrogen, NuPAGE SYSTEM). The run was performed at 200 V and 17 mA, using the NuPAGE® MOPS Running Buffer and stopped after 1 hour. For silver staining the amount of loaded proteins was 1 µg while for Coomassie Brilliant Blue staining 10 µg was used.

Gel-based protein identification: In-gel protein digestion

Seventeen protein bands were selected from the upper fraction 6.4% for protein identification. The gel bands were excised using a sterile scalpel and transferred to 0.5 mL eppendorf tubes. The protein bands were dehydrated with 100 µl of acetonitrile (ACN), rehydrated with 100 µl of 50 mM ammonium bicarbonate (ABC), and dehydrated again in ACN. These steps of dehydration/rehydration were performed several times until the bands were completely destained. Afterwards, the bands were dried in a SpeedVac and the proteins from the bands were digested with 0.1 µg/µl trypsin in the presence of 100 µl buffer containing 25 mM ammonium bicarbonate and 10% ACN. The samples were incubated at 37° C overnight.

Gel-based protein identification: Peptide extraction from gel

The peptides were extracted from the gel by treating the bands with the following solutions: 50 µl of 5% ACN and 0.1% formic acid (FA), 50 µl of 10% ACN and 0.1% FA and 50 µl of 95% ACN and 5% FA. Between each treatment the samples were vortexed, spinned down, incubated in a sonicator for 3 min and the supernatants transferred to new tubes. Thereafter the samples were dried in a SpeedVac. Salt contaminants were removed from the peptide samples by using a micro scale C18 sample preparation column (ZipTipµC18). Peptides were solubilized with 10µLof 5% ACN and 0.1% FA. ZipTipµC18 were attached onto a 10µL pipettor, hydrated five times with 10 µL of 100% ACN and 0.1% FA. Peptides were then loaded by aspirating and dispensing the resuspended digests through the ZipTips at least eight times to ensure maximal binding on the column and salt removal. Finally, the peptides were eluted by adding 3 µl of 60% ACN and 0.1% FA to the column. Subsequently, the samples were gently dried for 2 hour in a vacuum operator and the peptides were dissolved in 10 µl of 0.1% FA and 5% ACN.

Mass spectrometry analysis used for gel-based protein identification

The HPLC-MS/MS analysis was performed on a MicroTof-Q (Bruker). Five microliter of the peptide solution were injected and separated on an Ultimate 3000 (Dionex/Thermo Scientific) machine. The samples were separated using as buffer A: 99.9% MilliQ (MQ) water and 0.1% FA and as B buffer: 99.9% ACN and 0.1% FA. The column used was a Dionex Acclaim Pepmap 100, C18 peptide reverse phase column, 3 µm particle size and 100 Å pore size. Samples were eluted at a flow rate of 0.3 µL/min with a splitter ratio of 1/1000 using a gradient of 5% to 45% B for 42 min followed by a wash step of 5 min at 95% B ending with a re-equilibration step of 13 min at 5%. The MicroTof-Q was operated in nano-online positive ion mode with a nanospray voltage of 22 kV and a source temperature of 130° C. Bruker Daltonics Low Concentration Tune Mix was used as an external calibrant. The instrument was operated in data-dependent

acquisition (DDA) mode with a survey MS scan at a resolution of 15,000 for the mass range of m/z 400-1400 for precursor ions, followed by MS/MS scans of the top 3 most intense peaks with +2, +3 and +4 charges ions above a threshold ion count of 3,000 using normalized collision energy (NCE) of 10eV with an isolation window of 3.0 m/z, with a dynamic exclusion of 60s after 3 spectra. All data were acquired with Bruker Daltonics MicrotofControl 3.0. Peak detection and conversion to "mgf"-files was performed using MS Convert from Proteo Wizard 3.0.3631 software, with the following filter: ChargeStatePredictor 4 1 0.9. The mgf -files were submitted to Mascot (version 2.2.04) and a search database was performed using an in-house *Musa* database (MusaA_B) containing all the protein sequences of the published A and B genome (D'Hont et al., 2012; Davey et al., 2013), plus contaminant sequences (trypsin and keratin). Search parameters were set at: tryptic digestion, one miscleavage allowed, 100 ppm precursor mass tolerance and 0.5 Da for fragment ion tolerance with a fixed modification of cysteine carbamidomethylation and a variable modification of methionine oxidation. Mascot data were submitted to Scaffold (version 4.1) to generate a fasta file against the MusaA_B database. The fasta file sequences were annotated via Blas2go (Conesaet al., 2005) and their likely cellular compartment was further characterized by GO compartment searches. In addition, the protein sequences were subjected to the ARAMEMNON database (Schwackeet al., 2003) to search for transmembrane domains.

Gel-free protein identification: SDS removal and protein digestion

Twenty micrograms proteins from the plasma membrane fraction 7.2% (upper phase) and microsomal fraction, both solubilized in 500 µl buffer containing 4% SDS, 1% DTT and 100 mM Tris-HCl pH 7.6 were submitted to Microcon YM-30 filters (Millipore, Billerica, MA, USA) for SDS removal followed by protein digestion. First the plasma membrane and microsomal samples were mixed with 400 µL of 8 M urea in 0.1M Tris/HCl (pH 8.5) (i.e. UA buffer) and added to the filters. The filter devices were subsequently centrifuged at 13,000 rpm for 20 min and the flow-through was discarded. Next, the filters were washed with 200 µL of buffer UA, centrifuged at 13,000 rpm for 20 min and the flow-through was discarded. Two hundred µL UA buffer containing 20 mM DTT were added to the filter for 15 min incubation at room temperature followed by centrifugation at 13,000 rpm for 20 min and flow-through removal. Subsequently, 100 µL of UA buffer containing 0.05 M of IAA were added to the filters. After 30 min of incubation, the filter devices were centrifuged at 13,000 rpm for 20 min and the flow-through was discarded. Two hundred µL of a buffer containing 8 M urea in 0.1 M Tris/HCl (pH 8.0) (i.e. UB buffer) were added to filters and centrifuged at 13,000 rpm for 20 min and the flow-through were discarded. This step was repeated twice. Forty microliters of UB buffer containing endoproteinaseLys-C (enzyme-protein ratio of 1:50) were added into the filters, mixed and incubated overnight at room temperature. The next day, 120 µl of 40 mM ABC containing trypsin (enzyme-protein ratio 1:100) were added to the filters followed by incubation for 4 hours at 37° C. After digestion, the filters were centrifuged at 13,000 rpm for 20 min and the flow-through was kept. In addition, 50 µl of 0.5 M NaCl was added to the filters, centrifuged (13,000 rpm, 20 min) and the flow-through was collected.

Gel-free protein identification: Salt removal

The Thermo Scientific Pierce C18 Spin Columns were used to remove the salts from the samples. First, the digested samples (full proteome, microsomal fraction and plasma membrane fraction) were acidified with a buffer containing 2% TFA and 20 % ACN, (sample: buffer ratio 3:1) resulting in a final concentration of 0.5% TFA and 5% ACN. Second, the C18 columns were activated and equilibrated by adding 200 µl of 50% ACN and 200 µl 0.5% TFA in 5% ACN respectively, followed by centrifugation at 1,500 x g for 1 min after each step. Third, the samples were added to columns and centrifuged at 1,500 x g for 1 min. This step was repeated once to maximize the sample binding to the column. Fourth, the columns were washed with 200 µl of 0.5% TFA in 5% ACN, and centrifuged at 1,500 x g for 1 min. This step was repeated once to remove the maximum amount of salt contaminants. Finally, the samples were eluted from the column by applying twice 25 µl of 70% ACN and centrifuged at 1,500 x g for 1 min. Subsequently, the samples were dried in a vacuum operator until they were dry and the peptides were dissolved in 10 µl % of 0.1% FA and 5% ACN.

Mass spectrometry analysis used for gel-free protein identification

The UPLC-MS/MS analysis was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). One microliter of the samples, full proteome, microsomal and upper phase (plasma membrane fraction) was injected and separated on an Ultimate 3000 UPLC system (Dionex, Thermo Scientific, Netherlands) (Thermo Scientific™ Acclaim™ PepMap™ RSLC Nano-Trap Column with nanoViper™ Fittings, 3 µm

Particle Size). The samples were separated using as buffer A water 0.1% FA and buffer B 20% water, 80% ACN and 0.1% FA. The columns used were: an Easy-Spray column (Thermo Scientific), 15 cm x 50 µm ID, PepMap RSLC C18, 2 µm for the plasma membrane and microsomal samples, and an EASY-Spray column (Thermo Scientific), 50 cm x 75 µm ID, PepMap RSLC C18, 2 µm for the full proteome sample. Upper phase and microsomal samples were eluted from the 15 cm column at a flow rate of 300 µL/min using a gradient of 4% to 10% B for 6 min followed by a gradient of 10% to 35% B for 25 min, a gradient 35% to 65% B for 5 min and then a final elution and re-equilibration step at 95% and 5% B respectively for 10 min. The full proteome sample was eluted from the 50 cm column at a flow rate of 300 µL/min using a gradient of 4% to 12% B for 17 min followed by a gradient of 12% to 15% B for 3 min, a gradient 15% to 18% B for 45 min, a gradient 18% to 20% B for 40 min, a gradient 20% to 30% B for 65 min, a gradient 30% to 35% B for 30 min, a gradient 35% to 65% B for 5 min and then a final elution and re-equilibration step at 95% and 5% B respectively for 40 min.

The Q Exactive was operated in positive ion mode with a nanospray voltage of 1.5 kV and a source temperature of 250° C. ProteoMAss LTQ/FT-Hybrid ESI PosModeCalMix (MSCAL5-1EA SUPELCO, Sigma-Aldrich) was used as an external calibrant and the lock mass 445.12003 as an internal calibrant. The instrument was operated in data-dependent acquisition (DDA) mode with a survey MS scan at a resolution of 70,000 for the mass range of m/z 400-1600 for precursor ions, followed by MS/MS scans of the top 10 most intense peaks with +2, +3 and +4 charged ions above a threshold ion count of 16,000 at 35,000 resolution using normalized collision energy (NCE) of 25eV with an isolation window of 3.0 m/z, an apex trigger 5-15 sec and a dynamic exclusion of 10 s. All data were acquired with Xcalibur 2.2 software (Thermo Scientific). For identification, all raw data were converted into mgf files using Proteome Discoverer 1.3 (Thermo Scientific). The spectra were searched using Mascot (version 2.2.04) against our in-house *Musa* database (MusaA_B) containing all the protein sequences of the published A and B genome plus contaminant sequences (trypsin and keratin). Search parameter were set at: tryptic digestion, one miscleavage allowed, 10 ppm precursor mass tolerance and 0.02 Da for fragment ion tolerance with a fixed modification of cysteine carbamidomethylation and a variable modification of methionine oxidation. Next, the data were uploaded to Scaffold (version 3.6.3) to generate a fasta file of the proteins of interest. The filter conditions were 0.1% of protein false discovery and 0.9% of peptide false discovery. The fasta file sequences were annotated via Blas2go and their likely cellular compartment was further characterized by GO compartment searches.

RESULTS AND DISCUSSION

Analysis of the protein profiles from the total, soluble, microsomal, upper and lower fractions

The proteins isolated in the total, soluble, microsomal, upper and lower fractions from Cachaco roots were loaded in a one dimension SDS-PAGE gel (Figure 2a, b) to compare the protein profile of the upper phase to the total, microsomal and lower fractions. Using a more sensitive but non-mass spec compatible silver staining, we observed enrichment of some proteins in the upper phase (indicated by the red arrows in figure 2 a, b), which are not present or less abundant in the total, soluble, microsomal and lower fractions. Since the plasma membranes have the highest affinity for the PEG upper phase (Larsson, 1985), the proteins enriched in the upper phase have a high probability to be plasma membrane proteins. This is an indication that the aqueous-polymer two-phase system technique may have worked properly. To assess this, protein bands were extracted from a gel stained with Coomassie Brilliant Blue (Figure 4) for identification.

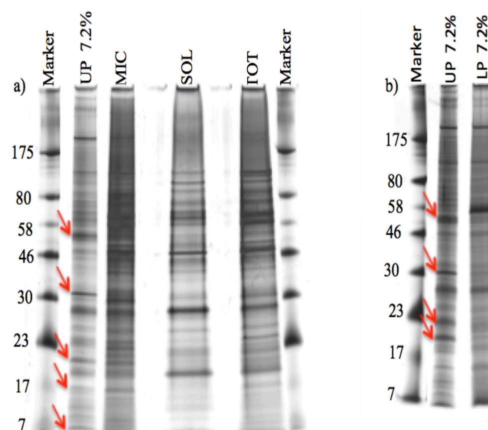

Figure 2 – Fractionation of proteins extracted from Cachaco roots. Two pre-casted NuPAGE®Novex®Bis-Tris Mini gels silver staining. Images obtained by ImageScanner from Amersham Biosciences. Each lane contains 1 *µg* of protein. a) Marker = ColourPlusprestained protein marker, UP = upper phase containing 7.2% (w/w) polymer concentration, MIC = microsomal fraction, SOL = soluble fraction, TOT = total fraction, b) UP = upper phase and LP= lower phase containing both 7.2% (w/w) polymer concentration.Red arrows indicate the proteins enriched in the upper phase (UP) 7.2% compared to total, soluble, microsomal and lower fractions.

Test of four polymer concentrations for the partition of proteins in the upper and lower phases

It is known that the polymer concentrations of PEG and dextran affect the protein partitioning in the aqueous-polymer two-phase system. Larsson (1985) reported that the majority of the membrane vesicles are in the upper phase when the polymer concentration is close to the critical concentration for the formation of the two phases. The increase in the polymer concentrations increases the hydrophobicity of the PEG-upper phase. As a result, the endomembranes especially the mitochondrion and endoplasmic membranes start to partition at the interface and the plasma membranes remain in the upper phase. Therefore we tested four different polymer concentrations: 6.2%, 6.4%, 6.8%, and 7.2% in order to compare the enrichment of proteins in the upper phases.

At the lowest polymer concentration, 6.2%, the upper and lower phases were not clearly distinct. As the polymer concentration increases, the difference in color between the two phases increases (Figure 3). The upper phase becomes more transparent and the lower phase darker. The 7.2% polymer concentration showed the most transparent color. The dark color is probably caused by the oxidation of polyphenol components, which are unwanted in the protein extraction.

Next, we applied the upper and lower phases (Figure 3) into a one dimension SDS-PAGE Coomassie gel to compare their protein profiles (Figure 4). The clear effect of polymer concentration we previously observed on the composition of the two phases was not observed at the protein profile level and irrespective of the polymer concentration there is still an overlap between the upper and lower phase.

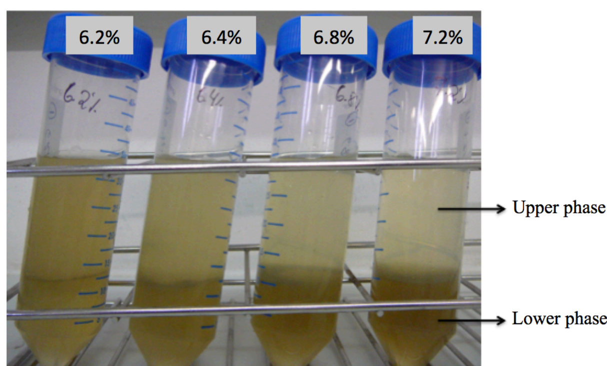

Figure 3 – Microsomal fraction extracted from Cachaco roots and added to four aqueous-polymer two-phase partitioning system containing the following PEG and dextran concentrations: 6.2% (w/w) PEG and 6.2% (w/w) dextran; 6.4% (w/w) PEG and 6.4% (w/w) dextran; 6.8% (w/w) PEG and 6.8% (w/w) dextran and 7.2% (w/w) PEG and 7.2% (w/w). Upper phase rich in PEG and lower phase rich in dextran formed after the first round of centrifugation at 1,800 x g for 5 min at 4 °C.

To have a better view on the composition of the proteins in the upper phase, we extracted some protein bands from the upper phase 6.4% for identification (indicated by red numbers in figure 4).

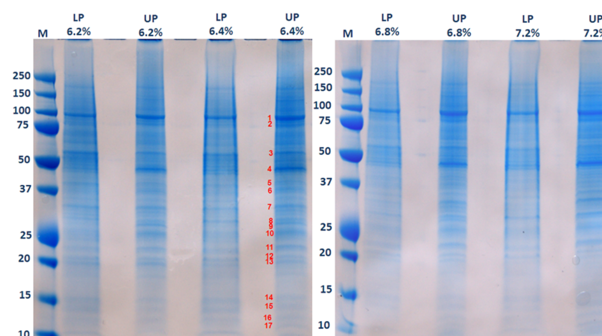

Figure 4 – Proteins from Cachaco roots isolated in the upper and lower phases (UP and LP) of four aqueous-polymer two-phase systems containing different polymer concentrations. Lower and upper phases 6.2% (w/w), 6.4% (w/w), 6.8% (w/w) and 7.2% (w/w) applied into two pre-casted NuPAGE®Novex®Bis-Tris Mini gels stained with Coomassie Brilliant Blue. Images obtained by ImageScanner from Amersham Biosciences. Each lane contains 10 µg of protein. Marker = ColourPlus prestained protein marker; MW = molecular weight; LP = lower phase; UP = upper phase. The numbers in red indicate the protein band number selected for identification.

Gel-based protein identification

Out of 17 protein bands extracted from the upper phase 6.4% (Figure 4), 7 bands led to the identification of 11 proteins but only one among them was predicted to be located at the plasma membrane: the aquaporin protein (KMMuB_chr11_G31730) (Table 1). The remaining proteins were predicted to be located at the cytoplasm and chloroplast. We also searched for the presence of transmembrane domains (TMD) and for the subcellular compartment. The presence of a transmembrane domain refers to the alpha helix of a transmembrane protein. This information can be useful for a protein being soluble or attached to a cellular membrane. Among the 11 proteins identified, the presence of transmembrane domains was detected only in the aquaporin protein (Table 1).

Table 1 – Proteins identified in the bands depicted in figure 4. The band where the protein was identified, accession number (MusaA_B database), protein name, number of unique peptides (Pept, determined by Mascot), highest peptide ion score (determined by Mascot), protein identification probability (determined by Scaffold), number of transmembrane domains (TMD; determined by Aramenon v8.0), and cellular compartment (CC determined by Blast2go). N = cellular compartment not identified, CHL = chloroplast, PM = plasma membrane, CYT = cytoplasm.

Band	Accession number	Protein name	Pept	Highest pept. ion score	Protein id. prob. (%)	TMD	CC
1,2	KMMuB_chr6_G16464	domain Putative SEC14-like phosphoinositide-binding protein	1	38.8	95	0	N
5	GSMUA_Achr11T04800_001 KMMuB_chr11_G32129	pto-interacting protein 1	2	32.2	99.8	0	N
5	GSMUA_Achr2T06110_001 KMMuB_chr2_G03648	quinone-oxidoreductase homo-log	2	33.9	91.4	0	N
7	KMMuB_chr3_G05416	peroxidase	4	33.9	100	0	N
8	GSMUA_Achr4T05250_001 KMMuB_chr4_G08857	lignin-forming anionic peroxi-dase	2	33.6	91.9	0	N
8,9	KMMuB_chr1_G00274	remorin	2	33	85.6	0	CHL
8,9	KMMuB_chr11_G31730	Putative PIP-type aquaporin/ small solute channel	2	34	98.6	6	PM
9	GSMUA_Achr2T15480_001 KMMuB_chr2_G04461	peroxidase 52	2	33.5	99.8	0	N
11	GSMUA_Achr5T26440_001	flavoproteinwrbA	2	31.8	100	0	N
11	GSMUA_Achr11T20550_001 KMMuB_chr5_G14552	60S ribosomal protein L18-2	6	32.7	90.5	0	CYT
11	GSMUA_Achr11T20440_001 KMMuB_chr11_G33855	glutathione S-transferase 3	2	32.7	90.5	0	CHL

The low numbers of proteins identified via the gel-based approach is linked to the low number of protein bands extracted from the gel, the percentage of identification (42%) is possibly due to protein losses during Coomassie removal from the proteins to avoid interferences during mass spectrometric analysis and protein extraction from the gel plugs (Shevchenko et al., 1996). Furthermore, this is a time consuming approach, which might have issues with reproducibility. Nevertheless, this approach is useful to visualize the protein profiles from different samples.

Gel-free protein identification

Considering the limitations of the gel-based approach for protein isolation and identification, the upper phase was additionally analyzed via a LC-MS/MS new generation mass spectrometer orbitrap QExactive. Along with the upper phase fraction (plasma membrane fraction), we also examined a full proteome fraction and a microsomal fraction for the presence of plasma membrane proteins.

In the upper fraction, a total of 502 proteins were identified, 239 were annotated, 123 were predicted to be located at the plasma membrane and at other cellular compartments, and 25 were predicted to possess transmembrane domains (TMD). In the microsomal fraction, a total of 895 proteins were identified, 431 were annotated, 219 were predicted to be located at the plasma membrane and at other cellular compartments, and 52 were predicted to possess TMD. In the full proteome fraction, a total of 633 proteins were identified, 296 were annotated, 148 were predicted to be located at the plasma membrane and at other cellular compartments, and 27 were predicted to possess TMD (Figure 5). From a total of 242 proteins predicted to be plasma membrane proteins, 59 were unique for the microsomal fraction, 14 for the full proteome fraction, and twofor the upper fraction (Figure 5 c).

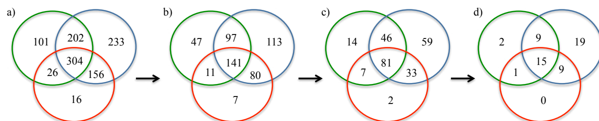

Figure 5 - Quantitative proteomics workflow. a) 1.037 proteins identified, b) 496 proteins annotated via blast2go, c) 242 proteins predicted to be located at the plasma membrane and in other cellular compartments, d) 55 proteins predicted to possess TMD. Green = full proteome, Blue = microsomal fraction, Red = plasma membrane fraction.

Among the plasma membrane proteins identified, we found 4 isoforms of aquaporins (PIP1-1, PIP1-2, PIP2-2 PIP2-6) in the microsomal fraction, 2 isoforms (PIP1-1 and PIP1-2) in the upper fraction and one isoform (PIP-2) in the full proteome. Other plasma membrane proteins such as cellulose synthase, cation-transporting ATPase, ABC transporter family member 2, and plasma membrane ATPase 4, pleiotropic drug resistance protein 3 (ABC transporter), high affinity nitrate transporter were identified only in the microsomal fraction.

Our results showed that the aqueous-polymer two-phase system technique is not the most useful technique to identify as many plasma membrane proteins as possible. Additionally, the application of this technique encounters the following difficulties: (i) it requires a significant amount of tissue (50 g) for extraction, (ii) optimization before application and, (ii) the protocol is time consuming. On the other hand, the protocol for the extraction of the full proteome and the microsomal fractions are more straightforward and do not need to be optimized for every plant tissue.

Despite the advantage of extracting a full proteome fraction and being able to characterize "the full proteome" in one step, the microsomal fractions yielded 28 proteins with TMD predicted to be located at the plasma membrane which were not identified in the full proteome.

We observed that the microsomal fraction, initially thought to be too complex and not sufficiently pure for plasma membrane protein identification (Vertommenet al., 2011), proved to be the most useful fraction to isolate plasma membrane proteins and not too complex for the current generation of mass spectrometers.

CONCLUSIONS

In this study, we described the isolation of plasma membrane proteins from banana roots using 4 different strategies. We first conclude that the gel-based approach was useful to visualize the proteins but not very efficient for the identification of proteins. Subsequently, we analyzed the upper fraction (plasma membrane fraction) via a gel-free approach and compared the amount of plasma membrane proteins identified with the ones identified in a full proteome and microsomal fractions. The microsomal fraction yielded the highest number (227) of identified plasma membrane proteins. Therefore, we finally conclude that the use of the microsomal fraction is the most efficient strategy to isolate plasma membrane proteins. We believe that the microsomal fraction is a valuable tool to study plasma membrane proteins. This is due to the advent of a high throughput mass spectrometry with high resolution and fast MS/MSMS cycle able to identify a substantial amount of plasma membrane proteins from the bulk of total membrane proteins (microsomal fraction). This result opens a new strategy for the characterization of plasma membrane proteins.

ACKNOWLEDGEMENTS

Suzana Garcia is supported by a Ph.D. grant of the National Council for Scientific and Technological Development (CNPq), Brazil. Financial support from the Belgian Directorate-General for Development Cooperation (DGDC) and Bioversity International 'ITC characterization' is gratefully acknowledged.

The authors would like to thank Annick De Troyer and Wesley Vermaelen for technical assistance.

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