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Extraction and fractionation of subproteome from root tips of *Hyoscyamus albus*

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ABSTRACT

Identification and quantification of different metabolites under stress, especially protein, is a vital way to understand plant adaptation mechanism. We established an efficient protein extraction method from the tiny amount (100 mg) of root tips of non-model medicinal plant *Hyoscyamus albus*, using bead-beating cell disruption, TRIzol extraction, and sequential chemical protein solubilization. *H. albus* is very well known for biosynthesized of different secondary metabolites like hyoscyamine, tropane alkaloids and scopolamine. Our method is rational for sample preparation even in small-scale proteomics of recalcitrant tissue and allows proficient, reproducible and impurity-free protein extraction. This method allows high-quality 2DE in mini-gel format (25 µg of protein/gel) for hydrophilic and hydrophobic sub-proteomes and is compatible to high-sensitive matrix-assisted laser/desorption ionization quadrupole ion trap time-of-flight (MALDI-QIT-TOF) mass spectrometry (MS). A protocol using TRIzol is more effective and reproducible to sequential chemical extraction of both hydrophilic and hydrophobic membrane proteins. We also demonstrated cell disrupted together with dithiothreitol **(**DTT) and polyvinylpolypyrrolidone (PVPP) is more useful to prevent polymerization of the phenolic compound than commonly used added DTT and PVPP with TRIzol reagent. Despite the unavailability of genomic sequence database, the efficacy of the protocol was also confirmed by MS/MS ion searches.

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Introduction

Nowadays, 2-DE and MS-based proteomics is a promising approach to identify enzymes and protein factors involved in various metabolisms, which are essential for plant adaptation under different environmental stress conditions. Proteins isolated from plant tissues, especially recalcitrant medicinal plants, are generally tough to resolve by 2-DE and to apply downstream MS analysis due to the presence of abundant interfering materials, such as charged metabolites, phenolics, polysaccharides, oxidative enzymes, and proteases (Aghaei and Komatsu, 2013; Isaacson *et al.*, 2006; Faurobert *et al.*, 2007).

Besides, the proteomic analysis of hydrophobic proteins such as membrane proteins based on 2-DE MS is complicated, due to content of low abundance membrane proteins as well as their amphipathic (containing regions that are hydrophobic and hydrophilic) nature, (Santoni *et al.*, 2000a; Santoni *et al.*, 2000b). Notably, most of the hydrophobic proteins are less soluble in the isoelectric focusing (IEF) sample buffer and precipitate at their isoelectric point (Santoni *et al.*, 2000a). In recent years, a significant effort has been paid to improve the enriching, extracting and partitioning the membrane proteins for proteomic analysis. Despite substantial recent progress, however, the analysis of plant proteins including membrane proteins, remains challenging, especially with respect to the sample preparation of recalcitrant non-model medicinal plant tissues. Lack of genomic sequence databases is another substantial impediment in medicinal plant proteomics since numbers of high-quality MS/MS

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spectra are required for cross-species database search and expressed sequence tag (EST) search (Rellán-Álvarez *et al.*, 2010; López-Millán *et al.*, 2013). Thus, efficient and optimized extraction method is to be considered as one of the vital steps for recovering the highest amount of reliable proteomic information.

The medicinal plant *H. albus*, belonging to Solanaceae family, is a rich source of alkaloids, namely tropane alkaloids of pharmaceutical interest, such as hyoscyamine, scopolamine, and their biosynthetic intermediates (Shimomura *et al.*, 1991). Unlike model plants such as *Arabidopsis thaliana*, *Solanum tuberosum* or *Medicago truncatula*, very few reports have been published on medicinal plant proteomics (Aghaei and Komatsu, 2013; Jacobs *et al.*, 2005; Wu *et al.*, 2012). Besides, medium or large-scale extractions are commonly used in omics studies that required large amounts of sample, that are generally unattainable for rare plant species, or when holistic, organ- or tissue-specific analyses need to be made. Our study on the proteomic analysis of cultured hairy root tips of *H. albus* has confronted precisely the difficulties as mentioned earlier with the tiny amount of tissue. So far, no successful proteomic analysis had been performed using any tropane alkaloid-producing plants including *H. albus*. In this study, we have used *H. albus* as a model of the recalcitrant plant. Publications on use of TRIzol method for plant proteomics are rare until now. Thus, it should be tested whether this method also works in medicinal plants and whether it requires modification and optimization, including cell disruption step.

In order to remove interfering materials and obtain high-quality 2D-gel profiles, protein extraction by Trichloroacetic (TCA) acetone or phenol-ammonium acetate/methanol has been commonly used for recalcitrant plant tissues (Isaacson *et al.*, 2006; Wang *et al.*, 2008). However, TCA acetone method alone is usually ineffective to remove such the interfering materials, and phenol-ammonium acetate/methanol method is a laborious and time-consuming method, which yields a relatively low amount of protein due to multiple steps in the extraction procedure. Acid guanidium thiocyanate-phenol-chloroform (AGPC) method using TRIzol reagent, initially developed for RNA isolation (Chomczynski and Sacchi, 1987). The same protocol has been employed for protein sample preparation from recalcitrant plants and algae (Lee and Lo, 2008; Wong *et al.*, 2006; Xiong *et al.*, 2011). It has recently been confirmed that no artificial modifications (e.g., carbamylation under the presence of urea) occur during TRIzol extraction (Young and Truman, 2012).

Here, we present an efficient subproteome fractionation protocol of hydrophilic and hydrophobic proteins from recalcitrant non-model medicinal plant root tissues, using bead-beating cell disruption, a modified protein extraction protocol with TRIzol reagent, and sequential chemical protein solubilization.

Methodology

Root culture and sample Collection

The hairy roots of *Hyoscyamus albus* L have been used in this study were cultured accordingly (Higa *et al.*, 2008). Twelve days old root tips (2 cm in length) were collected that was pre-cultivated in the standard liquid B5 medium (Gamborg *et al.*, 1968) containing one (1) % sucrose for two (2) weeks and then the medium was exchanged for the same medium, followed by further culture for 5 days. A 100 mL conical flask containing 25 mL of liquid medium was used for maintaining the culture, which was incubated on a rotary shaker at 25 °C with agitation at 80 rpm. Finally, root tips were harvested and kept at –80 °C until used.

Extraction of protein

Frozen root tips (100 mg fresh weight) with or without 20% w/w DTT (Bio-Rad, Hercules, CA, USA) and 10% w/w polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich, St. Louis, MO, USA) were disrupted for 1 min using a bead-beating apparatus (ShakeMaster Auto ver 1.5, BMS, Tokyo, Japan) equipped with pre-chilled bullet-shaped stainless bead crushers (SK-100-D10, Tokken, Inc., Chiba, Japan) and 2 mL stainless tubes (Tokken, Inc., Chiba, Japan), that were set in an aluminum block (25 well Master Rack for 1.5/2.0 mL tubes, BMS, Tokyo, Japan). After the cell disruption, 1 mL of TRIzol reagent (Invitrogen, Boston, MA, USA) with or without 20% w/w DTT and 10% w/w PVPP was mixed to the tube. The tube was shaken vigorously by hand for 10-20 seconds and incubated at room temperature for 5 mins, and then 0.2 mL of chloroform was added. After that, the tube was again strongly agitated for 15 seconds and allowed 2-3 min for incubation at ambient temperature, then centrifuged (12,000 x g, 15 min, at 4 °C) to differentiated into a lower organic phenol-chloroform phase, interphase, and an upper aqueous phase (Figure. 2A). The upper watery phase was collected to a new tube. Ethanol (0.3 fold volumes) was added to the remaining interphase and organic phase, mixed by inversion for 3-5 times, incubated at room temperature for 2-3 min and centrifuged (2,000 x g, 5 min, at 4 °C) separating the DNA pellet and the phenol-ethanol supernatant. The phenol-ethanol supernatant was dispensed to 1.5 mL low-binding PTX tubes (Progress Tube, IEDA Trading Corp., Tokyo, Japan). Then, 1.5 fold volume of isopropanol was added to each tube and incubated for 10 min at ambient temperature. The mixture was centrifuged (12,000 x g, 10 min, at 4 °C) separating the protein pellet and the phenol-ethanol/isopropanol supernatant. For SDS-PAGE analysis of proteins in the aqueous phase, DNA pellets, and phenol-ethanol/isopropanol supernatant were separately transferred to new tubes. Each protein pellet

was incubated with 1 mL of 300 mM guanidium hydrochloride in 95% v/v ethanol for 10 min at room temperature, and centrifuged (12,000 x g, 10 min, at 4 °C). This washing step was repeated three times.

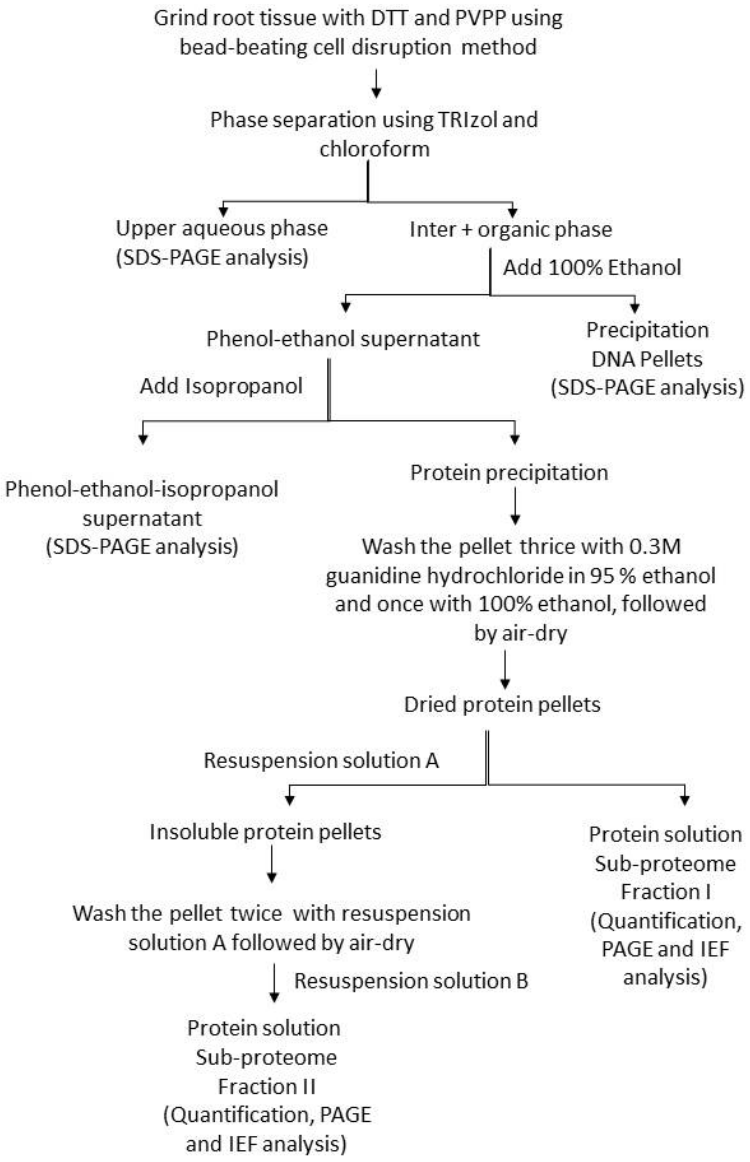

Fig. 1 Schematic Diagram of Phase Separation of *H. albus* Root Proteins by AGPC Method with TRIzol Reagent

The resulting pellet was allowed for incubation with 1 mL ethanol for 10 min at room temperature, centrifuged (12,000 x g, 10 min, at 4 °C), and the tubes with pellets were placed on the bench with the lids open to dry for 5-10 min (Figure 1).

Protein resolubilization

Proteins were extracted sequentially in a two steps protocol, as follows. Firstly, the protein pellet was dissolved in 60 µL of resuspension solution A (8 M urea, 50 mM DTT, 2% w/v CHAPS, 0.2% v/v carrier ampholyte, 0.001% w/v bromophenol blue), and homogenized with a plastic pestle, sonicated for 1 min in the ice-cold cup horn-type sonicator Astrason Ultrasonic Processor XL2020 (Misonix, NY, USA) at output level 4, and incubated for 3 h at room temperature, centrifuged (12,000 x g, 10 min, at 4°C). The supernatant was collected in new tubes and the remain insoluble pellet was washed twice by the above-mentioned resuspension solution A and finally dissolved into 60 µL of resuspension solution B (6 M urea, 2 M thiourea, 2 mM TBP, 2% SB3-10, 1% ASB-14, and 100mM DTT) which was designed for especially solubilizing hydrophobic membrane protein (Cordwell, 2008) and homogenized with a plastic pestle, incubated overnight at room temperature, centrifuged (12,000 x g, 10 min, at room temperature).

The aqueous phase and phenol-ethanol/isopropanol supernatant were dialysed against deionized water for 48 h at 4 °C, using Spectra/Por® dialysis membrane (MWCO 3,500), and concentrated (dehydrated) with Spectra/Gel® absorbent as described previously (Yamaguchi, 2011). The dehydrated sample was re-suspended in 120 µL of 1 x Laemmle buffer. All samples were incubated at 95 °C for 5 min, centrifuged at 15,000 x g for 3-4 min to remove insoluble materials, and loaded to each lane (10 µL/lane) for electrophoreses.

Protein quantification

The amount of protein in resuspension solution A was measured by a modified Bradford assay (Quick Start Protein Assay Kit, Bio-Rad, Hercules, CA, USA), while protein concentration in resuspension solution B was measured by RC DC (RC DC™ Protein Assay Kit, Bio-Rad, Hercules, CA, USA) method, in both cases BSA used as a standard.

SDS-PAGE

SDS-PAGE was performed using Mini-Protean TGX precast gel (Any kD, 10-well comb, Bio-Rad, Hercules, CA, USA) and Precision Plus Protein Dual Xtra Standards (Bio-Rad, Carlsbad, CA, USA) molecular weight marker as described previously (Yamaguchi, 2011).

2-D gel electrophoresis

The first dimensional IEF separation was carried out using 7 cm Ready Strip IPG Strips (Bio-Rad, Hercules, CA, USA) with a linear pH gradient pH 3-10 or pH 5-8 on a Protean IEF Cell (Bio-Rad, Hercules, CA, USA). The strips were passively rehydrated for 12 h at 20 °C in 125 µl of rehydration buffer containing protein sample (25 µg/strip). For sequential sub-proteome fractionation, two IEF solutions were used: IEF solution A (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.2% carrier ampholytes, 0.002% bromophenol blue dye and 50 mM DTT) and IEF solution B (6 M urea, 2 M thiourea, 2% w/v SB3-10, 1% w/v ASB-14, 0.2% carrier ampholytes, 0.002% bromophenol blue dye, and 100 mM DTT). 2DE was performed as described previously (Khandakar *et al.*, 2013), using Mini-Protean TGX precast gels (AnykD IPG/Prep, Bio-Rad, Hercules, CA, USA). Gels were stained with CBB R-250 (Bio-Rad, Hercules, CA, USA) or with Flamingo fluorescent stain (Bio-Rad, Hercules, CA, USA). Gel images for figure presentation were captured using a UV-vis laser scanner GELSCAN (iMeasure, Nagano, Japan). In linear pH gradient 3-10, most of the spots were focused in the central region of the 2D-gel; therefore, IPG strip with a narrower pH gradient 5-8 was chosen to improve resolution (Figure 2C-D).

Protein in-gel digestion and sample preparation for MALDI-QIT-TOF/MS

Protein spots were manually excised from 2D-gels using a spot image analyzer (FluoroPhoreStar 3000, Anatech, Tokyo, Japan) equipped with a gel picker (1.8-mm diameter). In-gel digestion, peptide extraction, and MALDI mass spectrometry were performed as previously described (Yamaguchi, 2011). MS and MS/MS spectra were obtained using a MALDI-QIT-TOF mass spectrometer (AXIMA Resonance, Shimadzu, Kyoto, Japan) in the positive mode. All the spectra were externally calibrated using human angiotensin II (m/z 1,046.54) and human ACTH fragment 18-39 (m/z 2,465.20) in a Proteo Mass Peptide & Protein MALDI-MS Calibration Kit (Sigma-Aldrich, St. Louis, MO, USA). MS/MS ion searches were performed using MASCOT version 2.3 (Matrix Science, London, UK) against SwissProt 2012_06 (536,489 sequences; 190,389,898 residues) and EST_Solanaceae 2012_04 (2,984,694 sequences; 560,101,884 residues) in our own MASCOT server. EST-encoded protein was identified by BLAST search (http://blast.ncbi.nlm.nih.gov/). Search parameters: enzyme, trypsin; fixed modifications, carbamidomethyl (Cys); variable modifications, oxidation (His, Trp, and Met); mass values, monoisotopic; peptide mass tolerance, ±0.5 Da; fragment mass tolerance, ±0.5 Da, max missed cleavage, 1.

Results and Discussion

According to the manufacturer's protocol of TRIzol reagent, proteins can be prepared from the interphase and organic phase, i.e. inter-organic phase (Figure 2A). To evaluate protein recovery by TRIzol reagent, protein content in the upper aqueous phase, interphase, organic phase, inter-organic phase, DNA pellets, phenol-ethanol/isopropanol supernatant phase, sub-proteome fraction A and B were analyzed by SDS-PAGE (Figure 2B). Proteins with low-mid-high molecular masses (7-200 kDa) were abundant in the sub-proteome fractions A (protein yield 5.03 ± 0.68 µg/g fresh weight), whereas proteins with mid-high masses (31-200 kDa) were abundant in the sub-proteome fractions B (protein yield 1.05 ± 0.33 µg/g fresh weight) (Figure 2B-D). Notably, the upper aqueous phase, DNA pellets, and phenol-ethanol/isopropanol supernatant phase did not contain a considerable amount of proteins (Figure 2B). Xiong *et al.* (2011) reported simultaneous isolation of DNA, RNA, and protein from different tissues (leaves, stems, and roots) of *M. truncatula*, suggesting that TRIzol protocol should be modified to optimize to this plant. In their method, the protein sample was prepared from the organic phase, and re-extracting proteins form the aqueous phase, but not form the interphase which was subjected to DNA preparation (not for protein preparation). In the case of *H. albus* root tips; however, the interphase contained a considerable amount and qualitative proteins (Figure 2B). Thus, the protein

sample prepared from the inter-organic phase was applied to further sub-proteome fractionations.

Remarkably, when DTT and PVPP were omitted at AGPC extraction or when frozen root tip powder thawed before the addition of TRIzol reagent with DTT and PVPP, disrupted root tips immediately turned brownish, resulting a small amount of pinkish-brown protein pellets and no distinct spots on 2DE (Data not shown). Even kept on ice or in cold room (0-4 °C), a brief thawing of the frozen powder provokes an immediate browning reaction; probably, polymerization of phenolic compounds by oxidative enzymes, and the brownish products critically interfere protein extraction and 2DE separation. Therefore, to obtain high-quality 2DE profile, it is imperative to disrupt frozen root tissue together with the reducing agent DTT and the phenolic absorbent PVPP and immediately to suspend in TRIzol reagent.

In principle, use of bead-beating and AGPC methods is quite reasonable for sample preparation in a small-scale proteomics of recalcitrant tissues, since physical disruption under liquid nitrogen in the closed stainless tubes allows efficient, reproducible, and contamination-free cell disruption (e.g., less keratin contamination) without loss of protein and protein modification/degradation, and the modified AGPC method enables efficient extraction with the strongest-known practical chaotropic (i.e., guanidium isothiocyanate) under the reducing condition: dissociating proteins and 2DE-interfering materials, also inactivating oxidative enzymes, proteases, and other unfavorable enzymes. Indeed, beneficial aspects of AGPG method, such as effective removal of interfering materials and no need for protease inhibitors, have been pointed out by Lee and Lo (2008).

To the best of our knowledge, 100-1,000 µg proteins per 2D-gel have been subjected for medicinal plant proteomics which required a high amount of initials fresh root materials. Unlike, our method requires only 100 mg fresh weight tissues for technical replicates of 2D mini gels (25 µg proteins/gel) in which consistent 214 ± 3 spots, and 139 ± 3 spots (3 replicates) were resolved with the samples solubilized in IEF solution A (Figure 2C) and B (Figure 2D) respectively. Further, to confirm that the protocol outlined above was compatible with MS analysis, ten protein spots (1 to 10) were randomly chosen (both prominent and faint spots in (Figure 2C-D) from the gels for analysis using MALDI-QIT-TOF mass spectrometer a unique hybrid MALDI type instrument, which enables high-sensitive MS and MS/MS analysis (sensitivity: > 500 amol). Despite the unavailability of genomic sequence database of *H. albus*, all of the spots were successfully identified by MS/MS ion searches.

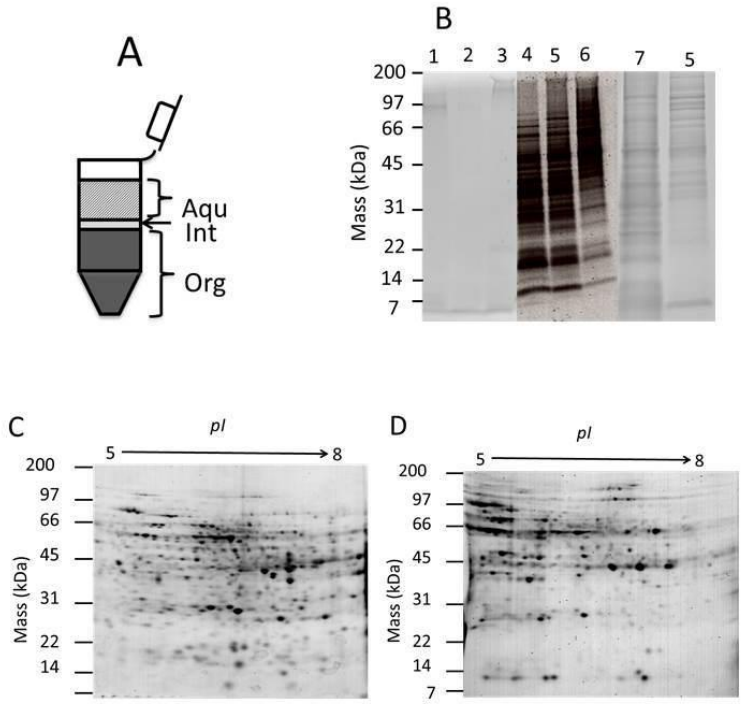

Fig. 2 Schematic Diagram of Phase Separation of *H. albus* root proteins by acid guanidium thiocyanate-phenol-chloroform (AGPC) method with TRIzol Reagent (A), SDS-PAGE Profiles of the proteins obtained by AGPC (B), and comparison of 2DE profiles of the proteins extracted by different methods (C-D). A, Aqu: aqueous (clear); Int: interphase (cloudy white); Org: organic phenol-chloroform phase (reddish-purple). B, Lane 1: proteins from the organic phase; lane 2: proteins from the inter-organic phase; lane 3: proteins from the interphase. C and D, Proteins prepared by AGPC from frozen root tip powder using TRIzol reagent with 2% w/v DTT and 1% w/v PVPP.

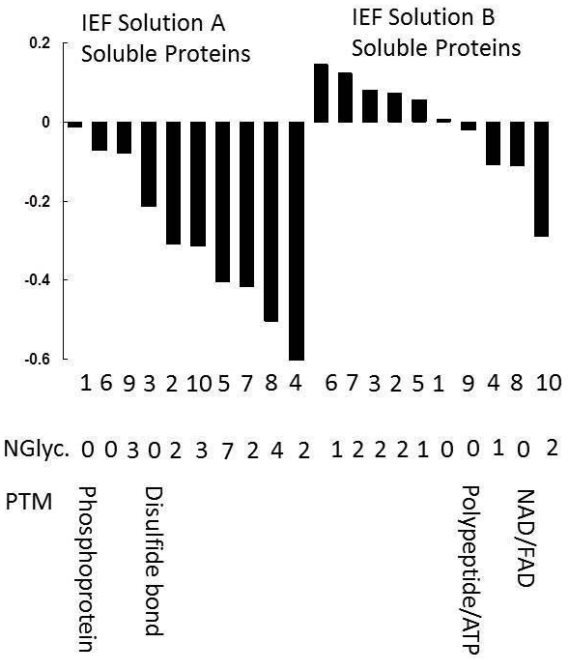

Fig. 3 Computer-aided hydropathy analysis (GRAVY values) of identified proteins from *H.albus* root tips. PTM: Post Translation Modification

Computer-aided hydropathy analysis (GRAVY values) of identified proteins suggested that sub-proteome fractions A enriched the cytoplasmic hydrophilic protein, whereas the sub-proteome fractions B contained both hydrophilic and hydrophobic proteins from *H. albus* root tips (**Figure 3**). Such solubility of the hydrophilic protein in detergent ASB-14/SB3-10 combined with strong chaotropic agent urea/thiourea possibly containing hydrophobic domains and/presence of post-translational modification. **Herbert *et al*. (1998)** noted that hydrophilic proteins have insoluble due to hydrophobic domains that are overridden by many hydrophilic residues. Post-translational modifications, such as myristoylation, palmitoylation or presence of a glycosylphosphatidylinositol anchor, are also affecting the solubilization of hydrophilic protein.

Conclusion

We have shown that our established protocol using TRIzol is more effective and reproducible to sequential chemical extraction of both hydrophilic and hydrophobic membrane proteins using a tiny amount (100 mg) of root tips tissue. We also demonstrated that cell disrupted together with DTT and PVPP is more useful to prevent polymerization of the phenolic compound than commonly used added DTT and PVPP with TRIzol reagent. Thus, our protocol (25 µg proteins per mini-gel) would contribute to minimizing the starting materials and facilitate proteomic characterization of *H. albus* root tips and other recalcitrant plant tissues. Finally, our method also showed compatible with downstream high-throughput MALDI-QIT-TOF mass spectrometer analysis.

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Conflict of interest

On behalf of all authors, the principal author declares that there is no conflict of interest.

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