Selective Enrichment Coupled with Proteomics to Identify S-acylated Plasma Membrane Proteins in *Arabidopsis*

Lijuan Zhou[1], Mowei Zhou[2], Marina A. Gritsenko[3], Gary Stacey[1,4*]

[1] Division of Plant Sciences, C.S. Bond Life Science Center, University of Missouri, Columbia, MO 65211, USA

[2] Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, 3335 Innovation Blvd, Richland, WA 99354, USA

[3] Biological Science Division, Pacific Northwest National Laboratory, 3335 Innovation Blvd, Richland, WA 99354, USA

[4] Division of Biochemistry, C.S. Bond Life Science Center, University of Missouri, Columbia,

MO 65211, USA

* Corresponding Author: Gary Stacey; Email: staceyg@missouri.edu

Abstract

Protein S-acylation, predominately in the form of palmitoylation, is a reversible lipid post-translational modification (PTM) on cysteines that plays important roles in protein localization, trafficking, activity, and complex assembly. The functions and regulatory mechanisms of S-acylation have been extensively studied in mammals, owing to remarkable development of the high-resolution proteomics and the discovery of the S-acylation-related enzymes. However, the advance of S-acylation studies in plants lags farther behind than that in mammals, mainly due to the lack of knowledge about proteins responsible for this process, such as protein acyltransferases (PATs) and their substrates. In this article, a systematic protocol to study global S-acylation in *Arabidopsis* seedlings is described. The procedures are presented in detail, including preparation of *Arabidopsis* seedlings, enrichment of plasma membrane (PM) proteins, followed by enrichment of S-acylated proteins/peptides based on the acyl-biotin exchange (ABE) method, and large-scale identification of S-acylated proteins/peptides via mass spectrometry. This approach enables researchers to study S-acylation of PM proteins in plants in a systematic and straightforward way.

Basic protocol 1: Preparation of *Arabidopsis* seedling materials

Basic protocol 2: Isolation and enrichment of plasma membrane proteins

Support protocol 1: Determination of protein concentration using BCA assay

Basic Protocol 3: Enrichment of S-acylated proteins by ABE method

Support protocol 2: Protein precipitation by methanol/chloroform method

Basic Protocol 4: Trypsin digestion and proteomic analysis

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Alternate protocol 1: Pre-resin digestion and peptide level enrichment

Keywords: S-acylation, palmitoylation, plasma membrane proteins, acyl-biotin exchange (ABE), proteomics

INTRODUCTION

Protein S-acylation, whereby a 16-carbon (palmitate) or 18-carbon (stearic acid) fatty acid chain is covalently linked to a cysteine residue through a thioester bond, is a reversable post-translational modification that makes it special among lipid modifications (Turnbull and Hemsley, 2017). The S-acylation state of proteins can dynamically regulate protein-membrane affinity and thus impact a variety of biological events in eukaryotes; e.g., cellular signaling, metabolism, pathogenesis (Li et al., 2017). Although progress has been made toward understanding many aspects of S-acylation in yeast and mammals, little is known regarding the role or extent of S-acylation in plants. Identification and characterization of functional S-acylated proteins will enhance our understanding of the biological roles of S-acylation in plants.

In the last two decades, S-acylproteomic approaches have greatly accelerated the characterization of S-acylated proteins and enabled researchers to study the dynamics of protein S-acylation at a global level. An acyl-biotinyl exchange (ABE) method (Drisdel and Green, 2004) coupled with the mass spectrometry has been widely used to achieve proteomic profiling of S-acylated proteins and to validate individual protein S-acylation (Hemsley et al., 2013; Yang et al., 2010; Collins et al., 2017a). This current protocol reflects our efforts to optimize the ABE method for plants and to achieve a better enrichment in a facile manner. For example, while S-acylation of a large number of plasma membrane proteins (e.g., receptor-like kinases; RLK) may be of biological interest, they are difficult to detect due to their relative low expression levels. Considering this, we adopted the Brij-58 treatment method (Collins et al., 2017b) to enrich the PM proteins prior to ABE and proteomics. Through this approach, our protocol successfully identifies a number of PM proteins with potential S-acylation sites.

The basic protocol described here includes four parts, as shown in Figure 1: (i) methods for preparation of *Arabidopsis* seedling materials in Murashige and Skoog (MS) medium (Basic Protocol 1); (ii) isolation of total proteins from plant tissues and enrichment of plasma membrane proteins based on a simplified and detergent-dependent method (Basic Protocol 2); measurement of protein concentration using BCA assay (Support Protocol 1); (iii) optimized enrichment of S-acylated proteins based on the ABE method (Basic Protocol 3); protocol of protein precipitation by methanol/Chloroform method is described as Support Protocol 2; (iv) S-trap trypsin digestion and mass spectrometry-based identification of S-acylated proteins (Basic Protocol 4); Alternate protocol 1 presents an approach for pre-resin digestion and the peptide-level enrichment which allows the identification of specific sites of S-acylation.

[*Insert Figure 1 here]

Basic Protocol 1

PREPARATION OF ARABIDOPSIS SEEDLING MATERIALS

To obtain sufficient seedling materials for plasma membrane (PM) protein enrichment and the subsequent S-acylation enrichment, a practical method of germinating and growing

Arabidopsis on MS medium in a plant growth chamber is described. The protocol includes surface sterilization of seeds with bleach and a method of placing single seeds onto the MS agar medium rapidly and evenly. This protocol makes it possible to maintain consistency between genotypes and between different sets of experiments. This protocol also simplifies the biological treatment procedure if required.

Materials

Arabidopsis Col-0 seeds

Bleach solution (20% commercial bleach + 0.1% Triton X-100)

½ Murashige & Skoog (MS) agar plates (see recipe)

Sterile water

Square petri dish (100 × 100 mm, Fisher, cat. 08-757-11A)

Deep petri dish (25 × 100 mm, Fisher, cat. 08-757-10E)

Eppendorf microcentrifuge tubes (2 mL)

3D rotator (Fisher Scientific, cat. 260100F)

Mini centrifuge (BioExpress)

Laminar flow hood (Labconco)

Plant growth chamber (22°C, 16-hr light/ 8-hr dark)

Forceps

Seed sterilization

1. Put the required amount of *Arabidopsis* Col-0 seeds in a 2 mL centrifuge tube.

   Note: To sufficiently sterilize the seeds, put no more than 300 µL volume of seeds in one tube. Basal volume of seeds will expand 2-fold during the sterilization procedures. Divide seeds into multiple tubes for sterilization if a large number of seeds are needed.

2. Sterilize seeds with 1 mL bleach solution for 15 min with gentle shaking on a 3D rotator at room temperature (RT).
3. After a short low-speed spin, discard the bleach solution with pipette.
4. Wash seeds thoroughly with 1 mL sterile water. Discard the water.
5. Repeat step 4 five more times. Add 1 mL sterile water to the seeds after the final wash.

   Note: Execute step 3 to 5 in the laminar flow hood to maintain aseptic conditions.

6. Place surface sterilized seeds at 4℃ for 3 days for vernalization to synchronize germination.

Seed germination and seedling growth

7. Plate seeds on ½ MS agar medium with 1 mL sterile pipette tips, spaced evenly.

   Note: To individually place seeds on the plate, pipette 150 µL of seeds suspension with a 1 mL pipette tip. This works better if the density of seeds in the seed suspension is adjusted with water to about 300 - 400 seeds / 150 µL. Hold up the plate, point the end of

the pipette to the plate gently and the seeds should flow out uniformly via surface tension. Put a white printing paper under the plate as a background to help distinguish seeds.

A density of 13 × 13 seeds / square plate (as shown in Fig.1) is preferred in this experiment. By doing this, the seedlings can grow healthily and, moreover, you can harvest plenty of seeding materials to accomplish the following protein enrichment procedures.

8. Allow the top agar or the plate lid to dry in the hood before covering the plates with lids. Seal with parafilm to prevent desiccation.
9. Place plates in the growth chamber vertically. Grow seedlings for one to two weeks at 22°C with a 16-hr light/ 8-hr dark cycle.

   Note: We can obtain about 2.5 g seedlings per square plate if seeds are placed with a density of 13 × 13 / square plate and grown for 2 weeks under normal conditions.
10. Using forceps, gently transfer the seedlings to deep petri dishes containing 40 mL sterile water. Place the transferred seedlings overnight in the growth chamber.

    Note: This step is necessary if a large number of seedlings is needed for protein extraction. The step also reduces wound response of seedlings, homogenizes samples, and benefits the following biological treatments.
11. Conduct biological treatment (biotic or abiotic stress, nutrition, light, etc.) as needed.
12. Take out of the seedlings from the treatment solutions, remove excess water with paper towel, and immediately freeze the samples in liquid nitrogen. Store at -80℃ until taking further steps.

    Note: We need approximately 10 g seedlings per sample for this experiment. Growth of 10 g seedlings requires 4 square plates if seedlings are planted for 2 weeks with suggested density.

Basic Protocol 2

ISOLATION AND ENRICHMENT OF PLASMA MEMBRANE PROTEINS

This protocol is used only if there is a specific interest in analyzing S-acylation of plasma membrane (PM) proteins. Enrichment of PM is essential in order to identify more S-acylated PM proteins, which often are present in whole cell extracts at low levels. A number of PM enrichment methods are available but most are time consuming and result in low recovery. As with many techniques, there is a trade-off of increased enrichment versus overall yield. Here, we employ a simplified enrichment procedure using detergent Brij-58 and ultracentrifugation (Collins et al., 2017b). The method is straightforward, easy to follow and reproducible. Our testing of various methods indicates that, while this method may not provide the greatest enrichment of PM proteins, it is sufficient for subsequent S-acylation analysis while maintaining good yield.

Materials

Liquid nitrogen

Homogenization buffer (H buffer, see recipe)

Brij-58 (Sigma, cat. P5884)

BCA protein Assay Kit (Pierce, cat. 23225)

Mortar and pestle

50 mL conical tubes (Thermofisher, cat. 339650)

Sorvall LYNX 6000 Centrifuge with F14-14 × 50cy fixed- angle rotor

Miracloth (Millipore, cat. 475855-1R)

Thick-walled polycarbonate ultracentrifuge tubes (Seton, PN-2005-30 mL)

Sorvall Discovery 100 SE ultracentrifuge with Surespin 630 rotor, 36 mL swinging buckets

Eppendorf safe-lock tubes (1.5 mL, Fisher, cat. 05-402-11)

Sorvall Discovery M150 SE ultracentrifuge with S45-A fixed angle rotor

Eppendorf microcentrifuge tubes (1.5 mL)

Paint brush with about 1 cm-long synthetic brush

Isolation of crude membrane

1. On the day of protein isolation, add proteinase inhibitors into pre-prepared homogenization buffer (H buffer).

   Note: Do not add DTT as a reducing reagent because DTT can break the thioether bond of the S-acylation. TCEP, a potent reducing agent that does not cleave thioester bonds, will be used to break disulfide bonds in the following protocol of enrichment of S-acylated proteins.

2. Dispense 20 mL H buffer into each 50 mL conical tube and keep on ice.

   Note: Calculate the amount of H buffer as 1 g tissue / 2 mL H buffer.

3. Take out of the seedlings from -80℃ freezer and keep in liquid nitrogen.

   Note: Ensure that you can protect your hands completely from liquid nitrogen while handling the materials within. Using a pair of forceps or wearing a water-proof, insulating glove can protect your skin.

4. Chill a mortar and pestle with liquid nitrogen.

5. Place a frozen *Arabidopsis* seedling sample (approximately 10 g) into the chilled mortar. Grind seedlings with pestle in liquid nitrogen for over 10 min until seedlings become a fine powder.

   Note: Keep the samples frozen by adding liquid nitrogen slowly during this procedure. To be consistent, use similar grinding force and time between different samples.

6. Scoop the finely ground frozen tissue into a 50 mL conical tube containing H buffer on ice.

   Note: From this point on, all steps must take place on ice or at 4°C.

7. Let seedling powder thaw in H buffer on ice. It takes about 1 hour to fully thaw and homogenize the cell lysate. Mix the homogenate well by gently inverting tubes every 10 min during the thawing process. Avoid vortex since it can break the membranes.

8. Spin total homogenate 10,000 × g at 4°C for 10 min.

9. Filter the supernatant through 1 layer of Miracloth into a new pre-chilled 50mL conical tube. Squeeze the Miracloth layer to collect all the cleared homogenate.

10. Spin filtered homogenate at 10,000 × g, 4°C for 20 min.

    Note: The supernatant of the homogenate is considered to be total proteins (T). Save about 100 µL of T fraction in a 1.5 mL microcentrifuge for further immunoblot analysis. Keep it on ice or store at -20°C.

11. Transfer the rest of the total homogenate to a 30 mL ultracentrifuge tube. Balance the tubes before being loaded to the swinging bucket.

    Note: In order to run an ultracentrifuge smoothly and safely at its operating speed, always follow the manufacturer's instruction manual, use the compatible ultracentrifuge tubes and load the proper sample volume. All opposing loads must balance within a certain weight as specified by the centrifuge manual.

12. Spin samples at 121,000 × g, for 4 hours at 4°C.

    Note: Speed (rpm) should be calculated from RCF (× g). Online tools can help with the calculation (e.g., https://www.sciencegateway.org/tools/rotor.htm). For SureSpin 630 rotor, speed of 121,000 × g equates to 260,000 rpm.

    Caution should be used when operating any high-speed centrifuge, regardless of brand. Training should be given to users before operating any ultracentrifuge.

13. After ultracentrifugation, gently transfer the tubes onto ice.

14. Carefully remove all the supernatant without disturbing the pellet.

    Note: Most of the cytosolic and solubilized proteins are present in the supernatant fraction (S_{121}), crude membrane proteins (CM) are enriched in the pellet. Save about 100 µL of S_{121} fraction in a 1.5 mL microcentrifuge for further immunoblot analysis. Keep it on ice or store at -20°C.

    Experiment can be paused here. Freeze membrane pellet at -20°C overnight.

15. Resuspend CM pellet in 50 µL H Buffer using a paint brush. To thoroughly resuspend the CM proteins, swirl the paint brush in CM in circles slowly and gently, avoid making bubbles. During this resuspension step, slowly add more H buffer in a 200 µL increment, up to an approximate total volume of 800 µL. Rinse paint brush with last addition.

    Note: The pellet appears to be thick and sticky because of the large amount of membrane lipids. It is necessary to add the H buffer in increments. It will be difficult to completely resuspend the pellet if too much buffer is added at once, small pellet pieces will appear and will be difficult to disperse.

16. Transfer re-suspended CM to a 1.5 mL microcentrifuge tube. Keep on ice.

17. Determine protein concentration for the CM fraction using the BCA protein assay kit (see Support protocol 1).

    Note: After completely resuspending the CM proteins, save about 50 µL in a 1.5 mL microcentrifuge for further immunoblot analysis. Keep it on ice or store at -20°C

Enrichment of plasma membrane (PM) proteins

18. On the same day as plasma membrane enrichment, add proteinase inhibitors into 30 mL pre-prepared H buffer; prepare 10 mL 1% Brij-58 buffer by dissolving 100 mg Brij-58 into 10 mL H buffer (10 mg Brij-58 / 1 mL H buffer) with vigorous shaking at RT; prepare 10 mL 0.2% Brij-58 buffer by diluting 2 mL 1% Brij-58 with H buffer. Chill all the buffers on ice.

19. Pipette 8 mg of CM proteins into a 1.5 mL Eppendorf safe-lock tube. Add calculated amount of H buffer and 1% Brij-58 buffer to achieve final Brij-58 amount to 0.15 µL of 0.2% Brij-58 buffer per µg CM protein and a total volume of 1.2 mL. Invert the tubes several times and incubate on ice for 30 min.

    Note: For example, if protein concentration of CM fraction is 15 µg/µL, Vol_{CM} = 533 µL; $Vol_{1\%\ Brij\text{-}58}$ = 240 µL; $Vol_{H\ buffer}$ = 1200 µL - 533 µL - 240 µL = 427 µL. Add them in the following order: CM fraction, H buffer, 1% Brij-58 buffer.

20. Balance samples with H buffer during the incubation.
21. Ultracentrifuge samples at 100,000 × g, for 30 min at 4°C (45,000 rpm for microcentrifuge rotor S45-A).
22. Discard the supernatant. For second Brij-58 incubation, resuspend the protein pellet in 40 µL 0.2% Brij-58 buffer with the paint brush. During the resuspension step, add more 0.2% Brij-58 buffer in a 200 µL increments to a total volume of 1.2 mL. Rinse paint brush with last addition. Incubate on ice for 30 min.
23. Balance samples with 0.2% Brij-58 buffer during the incubation.
24. Ultracentrifuge samples at 100,000 × g, for 30 min at 4°C.
25. Using the same method as Step 22 to resuspend enriched membrane pellet (ePM) in H buffer (no Brij-58) to a final volume of 400 µL.

    If ePM proteins are directly used for global proteomic analysis, two wash steps with H buffer should be conducted as step 22 to 24, 30 min incubation on ice is not needed.

26. Determine protein concentration for the final ePM proteins using the BCA protein assay kit (see Support protocol 1).
27. Pipette 2 mg ePM proteins to a new 2 mL microcentrifuge tube for the following S-acylation enrichment, keep the volumes consistent across samples by adjusting with H buffer.

    Note: The rest of the ePM proteins can be stored at -20℃ for further SDS-PAGE or immunoblot analysis.

28. Store ePM proteins at -80℃ until taking further steps.

Support Protocol 1

DETERMINATION OF PROTEIN CONCENTRATION USING BCA ASSAY

Quantitation of proteins is a common protocol. We specifically describe the BCA protein assay here because it is compatible with detergent (e.g. SDS).

Materials

Protein samples to be tested

BCA protein Assay Kit (Pierce, cat. 23225)

Distilled, deionized H_2O (ddH_2O)

96-well clear microplate (Fisher, cat. 07-200-95)

Microplate reader (Bio-Tek, Synergy HT)

1. Prepare 200 µL 1 µg/µL albumin (BSA) as a working standard.
2. Determine the total volume of BCA working reagent required. Prepare BCA working reagent by mixing reagent A and B at a ratio of A:B = 50:1. Mix well by inverting or vortex.

   Note: Volume of BCA working reagent required = (# standards + # unknown samples) × (# replicates) × 200 µL
3. For BSA standard, pipette 0 to 10 µL 1 µg/µL BSA into wells of the microplate in duplicate or triplicate, add ddH_2O to bring to 10 µL.
4. For samples to be tested, pipette 2 µL samples and 8 µL ddH_2O in duplicate or triplicate.

   Note: To make sure the amount of tested protein is in range of the BSA standards, dilute samples five or ten times before loading if the predicted concentrate of proteins is higher than 5 µg/µL.
5. Add 200 µL of the BCA working reagent to each well and mix the plate thoroughly on a plate shaker for 30 s.
6. Cover plate and incubate at 37℃ for 30min.
7. Cool plate to RT. Measure the absorbance at 562 nm on a microplate reader.
8. Prepare a standard curve based on the absorbance values of BSA standards in excel. Use the standard curve to determine the protein concentration of each unknown sample.

Basic Protocol 3

ENRICHMENT OF S-ACYLATED PROTEINS BY ACYL-BIOTIN EXCHANGE (ABE) METHOD

The protocol described here employs a Low-background ABE (LB-ABE) method that can be applied to any type of tissue and biological treatment and results in less background contaminants (Zhou et al., 2019). The protocol involves reduction of disulfide bonds by TCEP, free thiol blocking by N-Ethylmaleimide (NEM) and 2,2'-Dithiodipyridine (DTDP), acy-biotin exchange with HPDP-biotin, and capture of biotinylated proteins by streptavidin resin. It allows studies of the global S-acylproteome by proteomics and validation of the S-acylation state of a specific protein by western blot.

Materials

Methanol (Fisher, cat. A412-500)

Chloroform (Fisher, cat. C606SK-4)

2% SDS buffer (2SB buffer, see recipe)

0.5 M Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich, cat. C4706)

50 mM Tris-HCl pH 7.4 (Fisher, cat. BP152-10)

3.33 M N-Ethylmaleimide (NEM; Sigma-Aldrich, cat. 04259) in ethanol

50 mM 2,2'-Dithiodipyridine (DTDP; Sigma-Aldrich, cat. D5767) in DMF

2 M hydroxylamine (Hyd, Sigma-Aldrich, cat. 159417) pH 7.4, freshly made and keep on ice

1 mM EZ-Link™ HPDP-biotin (Thermofisher, cat. 21341)

High-capacity streptavidin agarose resin (Pierce, cat. 20357)

Dilution Buffer (see recipe)

Equilibration buffer (E buffer, see recipe)

Microcentrifuge tubes (2 mL, 1.5 mL)

Thermomixer (Eppendorf, cat. 5355)

Vortex mixer (Fisher Scientific, Votex-Genie 2, cat. 12-812)

3D rotator (Fisher Scientific, cat. 260100F)

Bench-top centrifuge (Fisher Scientific, accuSpin Micro 17)

Spin columns – snap cap (Pierce, cat. 69725)

Conduct the experiment at RT unless otherwise specified.

Protein reduction and free thiol blocking

1. Take out of the 2 mg ePM protein samples (400 µL in volume) from -80°C and thaw the proteins on ice.
2. Precipitate the proteins using the methanol/chloroform precipitation method (see Support Protocol 2).

   Note: This step will remove DNAs, RNAs, lipids, and small molecule metabolites.

   The volume of starting protein for the precipitation procedure should be no more than 200 µL to fit into 2 mL microcentrifuge tubes. Divide the 400 µL ePM protein sample into two 2 mL centrifuge tubes, 200 µL each, for precipitation. Combine the two aliquots in the process of precipitation (see Support Protocol 2).
3. To re-solubilize protein pellets, agitate the proteins in 50 µL 2SB buffer with a sterile 1 mL pipette tip. After breaking the big pellet pieces, add another 150 µL 2SB buffer. Incubate the tube in a Thermomixer at 1,200 rpm at 37°C until no obvious protein clumps appear. Add 200 µL 2SB buffer up to a 360 µL volume, mix well by inverting tubes.

   Note: To avoid clogging the pipette tip and to accelerate breaking the pellet pieces, cut 2 - 3 mm off the end of the pipette tip.

   To re-solubilize the protein well, it may take 20 min or longer using the Thermomixer. The step will reduce the time needed for re-solubilization in later steps since the protein amount will be largely decreased due to the loss in the precipitation steps.
4. Add 40 µL 0.5 M TCEP to a final concentration of 50 mM. Vortex and then incubate at RT for 60 min with rotation on a 3D rotator.
5. Remove excess TCEP by methanol/chloroform precipitation (see Support Protocol 2).
6. Re-solubilize protein pellets in 2SB buffer as described in step 3. The total volume of 2SB buffer in this step is 200 µL.

   Note: The final volume of 2SB buffer can be scaled up, but the volume will determine the number of aliquots for further protein precipitatation. More 2SB buffer may need more

aliquots for protein precipitatation, which leads to more operations and enhanced possibilities of losing protein.

7. Add 194 µL 50 mM Tris-HCl pH7.4 (97% v/v) and 6 µL 3.33 M NEM (3% v/v). Vortex and then incubate in the dark at 55 °C with shaking at 300 rpm in a Thermomixer for 60 min.

   Protect NEM from light.

8. Remove excess NEM by methanol/chloroform precipitation (see Support Protocol 2).
9. Re-solubilize protein pellets in 2SB buffer as described in step 3. The total volume of 2SB buffer in this step is 100 µL.
10. Add 100 µL 50 mM DTDP. Vortex and then incubate at RT for 60 min with rotation on a 3D rotator.
11. Remove excess NEM and DTDP by three sequential methanol/chloroform precipitations (see Support Protocol 2).

    Note: A complete removal of the free-cysteine blocking reagent (NEM and DTDP) is critical for enriching S-acylated thiols. You may be tempted to reduce the number of precipitations in order to reduce protein loss. However, experience indicates that sequential precipitations are needed as described.

    The experiment can be paused here. Stored the protein pellet at −80 °C overnight.

S-acylation sites labeling by Acyl-Biotin Exchange

12. Re-solubilize protein pellets in 2SB buffer as described in step 3. The total volume of 2SB buffer in this step is 400 µL.
13. Equally divide the protein solution into two 2 mL centrifuge tubes (200 µL each). Add the following reagents to each tube, respectively.

    a) experimental sample (Hyd+): 100 µL 2 M neutralized hydroxylamine pH 7.4 (Hyd), 100 µL 1 mM HPDP-biotin;

    b) control sample (Hyd-): 100 µL 50 mM Tris-HCl (pH 7.4), 100 µL 1 mM HPDP-biotin

    Incubation at RT for 60 min with rotation on a 3D rotator,

    Note: The control samples (Hyd-) absent of hydroxylamine are processed in parallel with the experimental sample (Hyd+) for all the following procedures. To effectively distinguish S-acyl-proteins from non-specific contaminant background, the Hyd- sample is necessary.

14. In the course of step 13, pre-equilibrate the streptavidin agarose resin. Each protein sample (including Hyd+ and Hyd-) needs at least 100 µL resin in storage solution (50 µL settled resin). Pre-equilibrate required total volume of resin.
    a) Equilibrate the resin to RT.
    b) Pipette the resin to spin columns. Place columns into collection tubes (use the collection tubes supplied with prepacked columns).

       Note: To sufficiently equilibrate the resin, transfer no more than 400 µL resin into one spin column.

    c) Centrifuge at 400 × g for 1 minute, discard storage solution from the collection tubes.

d) Add 600 µL Equilibration buffer (E buffer) on top of the resin bed. Press hard to close the snap cap, hold tightly to keep the column and collection tube leak-proof. Mix well by inverting 10 times.

Bottom cap is not needed when washing the resin with E buffer if the column and collection tube can be tightly connected. If the connection is not fluid-tight, buffer will leak to the collection tube from the bottom of the column by gravity. In this case, perform gentle pipetting instead of inverting to mix the solution well.

e) Centrifuge at 400 × g for 1 minute, discard E buffer from the collection tubes.

f) Repeat step 14.4 to 14.5 two more times.

g) Add 100 µL E buffer /100 µL bed resin to the column. Transfer the re-suspended resin to a new 2 mL tube by pipetting from top of the column.

h) Repeat step 14.7 to collect the remaining resin. Combine all the resin.

i) Equally divide the resin into new 2 mL centrifuge tubes according to the required number of samples.

15. Remove excess biotin-HPDP by two sequential methanol/chloroform precipitations (see Support Protocol 2).

Purification of biotinylated proteins with Streptavidin resin

16. Re-solubilize protein pellets in 50 µL 2SB buffer as described in step 3. Do not add more 2SB buffer.

17. Add 950 µL Dilution buffer to decrease the concentration of SDS to 0.1%. Vortex and then centrifuge at 16,000×g for 5 min at RT.

    Note: Save 50 µL supernatant in a new 1.5 mL centrifuge as input of the enrichment for further immunoblot analysis. Store at -20ºC.

18. Transfer the supernatant to a 2 mL centrifuge tube containing pre-equilibrated high-capacity streptavidin agarose resin (from step 14), and incubate at RT for 60 min with rotation on a 3D rotator.

    Note: Protein amount can't exceed the resin's binding capacity.

19. Transfer solution containing streptavidin resin to a new spin column that has been placed in a collection tube (use the collection tube supplied with prepacked column). Label the column. Centrifuge at 400×g for 1 min at RT, discard flow-through.

    Note: If the solution volume is such that the entire sample cannot be added at once, centrifuge the column to allow some of the solution to pass through, then add more protein solution.

20. Add 600 µL E buffer. Press hard to close the snap cap, hold tightly to keep the column and collection tube leak-tight. Wash the resin thoroughly by inverting 10 times and then centrifuge at 400 × g for 1 minute. Discard E buffer from the collection tube.

21. Repeat step 20 for five more times.

22. Place the column in a new 1.5 mL centrifuge tube (used as collection tube) and centrifuge at 400 × g for 2 min to remove excess E buffer. Discard the centrifuge tube.

23. Replace the bottom cap onto the column (use white tip supplied with prepacked column) and place the column into a new 1.5 mL centrifuge tube. Label the centrifuge tube.

24. To elute the enriched biotinylated proteins, add 100 µL 50 mM TCEP in E buffer to each column, incubate at 37ºC for 20min in a Thermomixer with rotation at 800 rpm, and then centrifuge tubes at 400×*g* for 2 min. Keep the flow-through.
25. Repeat step 24. Combine the flow-through.

    Note: Save 20 µL S-acylation enriched proteins in a 1.5 mL centrifuge tube and store in -20ºC for further immunoblot analysis. The success of the enrichment experiment can be validated by western blotting against a known, acylated protein marker (e.g. AtFLS2). In a successful experiment, when loading the same amount of the eluted proteins, the signal of the marker protein in the Hyd+ sample should be much stronger than the Hyd- sample (as shown in Figure 2A).
26. Precipitate proteins by the methanol/chloroform method (see Support Protocol 2).

    Note: The experiment can be paused here. Store the protein pellet at –80 ºC.

[*Insert Figure 2 here]

Support Protocol 2

PROTEIN PRECIPITATION BY METHANOL/CHLOROFORM METHOD

Some reagents may impair the downstream procedures. Removing interfering contaminants is critical to these steps. We choose to use the methanol/chloroform precipitation method since it does not require sample cooling, is simple to do, inexpensive, and time-saving. Protein precipitation with chloroform and methanol results in dry protein material with high recovery, free of salt and detergent (Fic et al., 2010).

Materials

Protein samples

Methanol (MeOH, Fisher, cat. A412-500)

Chloroform ($CHCl_3$, Fisher, cat. C606SK-4)

ddH_2O

Fume hood (Labconoco)

Vortex mixer (Fisher Scientific, Votex-Genie 2, cat. 12-812)

Bench-top centrifuge (Fisher Scientific, accuSpin Micro 17)

SpeedVac concentrator (Thermo, Savant DNA 120)

Note: All steps are conducted at RT in a fume hood. To sample of 200 µL starting volume,

The method can be scaled up or down. To simplify our operations, we perform the precipitation within 2 mL centrifuge tubes. For sample of 400 µL or more total volume, divide proteins into two or more centrifuge tubes, and precipitate separately. After step 5, combine the floating protein aliquots by pouring all protein solutions into one tube. Continue the following procedures from step 6.

1. Add 800 µL MeOH and mix well by vortex for 20 sec.
2. Add 200 µL $CHCl_3$ and mix well by vortex for 20 sec.

3. Add 600 µL ddH_2O and mix well by vortex for 30 sec. Sample should look cloudy.
4. Spin 2 min at 14,000g at RT.
5. Pipette off the upper layer leaving a small amount of upper phase behind.

   Note: Proteins are suspended between the two liquid phases: top phase, MeOH/H_2O; bottom phase, $CHCl_3$.
6. Add 1 mL of MeOH and mix well by inverting.
7. Spin 5 min at 16,000g at RT.
8. Carefully pipette off the supernatant as much as possible without disturbing the pellet.
9. Dry the proteins by Speed-Vac, avoid drying too long as this makes the pellet harder to re-solubilize.

Basic Protocol 4

TRYPSIN DIGESTION AND PROTEOMIC ANALYSIS

A variety of proteomic approaches and equipment can be used to analyze the control and S-acylated proteins, as long as they are compatible with the samples. By way of example, we present the specific methods we have used. In this protocol, protein trypsin digestion is performed by the suspension trap (S-Trap) method. S-trap digestion tolerates a high concentration of SDS, provides efficient digestion, and results in peptide samples ready for MS. Subsequently, the digested peptides are separated and identified using a Waters NanoAcquity liquid chromatograph (LC) coupled with a Thermo Orbitrap Fusion lumos high resolution mass spectrometer (MS).

Materials

20% Sodium Dodecyl Sulfate Solution (SDS, Invitrogen, AM9820)

1M Tris pH 8.0 (Invitrogen, AM9855G)

SDS Lysis Buffer (5% SDS, 50 mM Tris, pH 7.55)

500 mM Iodoacetamide (IAM, No-Weight Format Pierce, A39271)

20% Phosphoric Acid (RICCA Chemical Company, 5851-16)

Methanol Optima LC/MS Grade (Fisher Chemical, A456-212)

S-Trap Binding Buffer (90% Methanol, 100 mM Tris, pH 7.5)

Digestion Buffer (50 mM Tris, pH 8.0)

0.2% Formic Acid (FA, Sigma, F0607) in LC/MS Grade water (Fisher Chemical, W6-4)

0.2% Formic Acid in 50% Acetonitrile (ACN, Fisher Chemical, A955-4): LC/MS Grade water

0.1% Formic Acid in 3% Acetonitrile: LC/MS Grade water

Sequencing Grade Modified Trypsin (Promega, cat. V5111)

S-Trap™ micro spin columns (PROTIFI, Prod #: C02-micro-10)

Centrifuge 5415 D (Eppendorf)

ThermoMixer C (Eppendorf)

Vortex Genie-2 (Scientific Industries)

Fisherbrand 1.5 mL Microcentrifuge Tubes (Fisher Scientific, 02-681-331)

Speed-Vac Concentrator SC250EXP (Thermo Scientific)

Reversed phase liquid chromatography (RPLC) column (packed in-house): Phenomenex C18 3 µm particle, pore size 300 Å, column ID 75 µm, OD 360 µm, length about 60 cm

Online solid phase extraction (SPE) trap column: Phenomenex Jupiter C18 3 µm particle, column ID 150 µm, OD 360 µm, length about 5 cm

Mobile phase A (MPA): 0.1% FA in water; mobile phase B composition (MPB): 0.1% FA in ACN

Liquid chromatography (LC): Waters NanoAcquity or equivalent, dual pump for online desalting

Mass spectrometer (MS): Thermo Orbitrap Fusion lumos or other high-resolution mass spectrometers compatible with proteomics workflows

Note: Conduct the experiment at RT unless otherwise specified.

Alkylation and trypsin digestion

1. Resuspend protein pellet (~ 20 µg protein) in 50 µL SDS Lysis Buffer.

   Note: The initial volume of SDS Lysis Buffer can be larger if needed; adjust the volume of S-Trap Binding Buffer accordingly.

2. Centrifuge at 13,000 × g for 8 min. Transfer the supernatant to a new centrifuge tube.
3. Alkylate unlabeled cysteines by adding 1 µL 500mM IAM to a final concentration of 10 mM. Incubate in a Thermomixer in the dark, at 25°C for 45 min with 300 rpm shaking.
4. Add 5 µL 12% phosphoric acid to each 50 uL of the SDS Lysis Buffer for a final concentration of 1.2% phosphoric acid. Mix well by vortexing and then centrifuge solution via short spin.

   Note: This step is important as the protein trap binds at this pH.

5. Add 385 µL of S-Trap Binding Buffer to the each 55 uL of acidified SDS Lysis Buffer (total volume of 440 µL). Mix well by vortexing.

   Note: For starting volumes > 50 µL of SDS Lysis Buffer, maintain a 1:7 v:v ratio of SDS Lysis Buffer to S-Trap Binding Buffer.

6. Place the S-Trap micro spin column in a 1.5 mL microcentrifuge tube, add the acidified SDS Lysate/S-Trap Binding Buffer mixture onto the spin column.

   Note: No column pre-equilibration is necessary. Do not exceed volume of the spin column when loading solution. If the initial SDS lysate volume is higher, load the column multiple times until the full volume has been bound. To load 440 µL total solution, load 220 µL, centrifuge through, then repeat with the next 220 µL.

7. Centrifuge the spin column at 4,000 × g for 30 sec or longer until all SDS Lysate/S-Trap Binding Buffer has passed through the S-Trap column.

   Note: Proteins will be bound and trapped within the protein-trapping matrix of the spin column.

8. Wash captured protein by adding 150 µL S-Trap Binding Buffer and centrifuging the spin column at 4,000 × g for 30 sec.
9. Repeat wash step for three more times.
10. Transfer the S-Trap spin column to a new 1.5 mL microcentrifuge tube.

    The use of a new tube prevents contamination of the resulting digestion.
11. Add 20 µL of Digestion Buffer with Trypsin at 1:10 (trypsin: protein, w/w) into the top of the spin column.

    Note: Trypsin should be not less than 0.75 µg. Ensure that there is no air bubble between the protease digesting solution and the protein trap.
12. Close the cap of the micro spin column loosely to limit evaporative loss.
13. Incubate for 1 hr at 47°C for Trypsin digestion. Do not shake.
14. Add 40 µL of Digestion Buffer to the S-Trap spin column containing the protease.
15. Centrifuge at 4,000 g for 60 sec to elute the peptides. This aqueous elution contains most peptides.
16. Add 40 µL of 0.2% FA in water to the S-Trap spin column. Centrifuge at 4,000 g for 60 sec.
17. Add 35 µL of 0.2% FA in 50% ACN: water. Centrifuge at 4,000 g for 60 sec.

    This elution assists in recovery of hydrophobic peptides.
18. Pool all eluted peptide solutions, dry down peptides in Speed-Vac Concentrator.
19. Resuspend peptides in 3% ACN, 0.1% FA in water at 0.1 µg/µL for MS analysis.

Proteomic analysis

20. Configure LC for 5 min online desalting in the SPE trap column with MPA at 5 µL/min.

    Note: The online desalting is achieved with dual pump LC configuration. After sample is injected onto the SPE trap column, the "wash" pump flows at 5 µL/min with MPA to desalt the sample. After desalting, the "wash" pump is diverted and the "analytical" pump flows into the SPE. The gradient starts and peptides are gradually eluted from the SPE into the analytical column. Please consult LC manufacturer for details on the setup.
21. Configure LC method for 150 min separation at 0.3 µL/min. A representative gradient table is listed in Table 1. % MPA = 100% - % MPB.

    [*Insert Table 1 here]
22. Configure data dependent acquisition for MS. Representative settings for Orbitrap instrument is listed below:

    Ion source settings: electrospray voltage 2.2 kV, s-lens RF 30%, capillary temperature 250 °C. No source gas.

    MS1 settings: 60k resolution, AGC target 4E5, maximum injection time 50 ms, scan range 400-2000 m/z, profile spectra.

    MS2 settings: 15k resolution, AGC target 1E5, maximum injection time 22 ms, isolation window 1 m/z, normalized collision energy 30±5, centroid spectra.

Data dependent acquisition: cycle time 3 seconds, intensity threshold 5E4, include charge state 2-6, dynamic exclusion of 45 seconds.

23. Inject 5 µL peptide solution (0.1 µg/µL) for each sample and collect MS data.
24. Process the data with proteomics software (e.g. MSGF+, MaxQuant, ProteomeDiscoverer, Byonic, PEAKS Studio, etc). Download protein FASTA from UniProt for all *Arabidopisis* protein sequences. Append common contaminant proteins sequences such as trypsin and human keratin.

    Note: Some proteomic software has a built-in function to add contaminant proteins. If not, manually add the proteins sequences in the FASTA.

25. Include the following dynamic modifications in the peptide search: carbamidomethyl (+57.0215) on Cys, N-ethylmaleimide (+125.0477) on Cys, dithidipyridine (+108.9986) on Cys, methionine oxidation, protein N-terminal acetylation, deamidation (Asn, Gln).
26. Peptides with carbamidomethyl (label with IAM) are most likely S-acylated in vivo. Example data is shown in Fig. 2B using Byonic software for peptide identification. Mass error tolerance is 10 ppm.

    Note: Non-S-acylated cysteines which are not effectively blocked by NEM and DTDP (step 7-11 in Basic Protocol 3) will be labeled by IAM. In addition, proteins with S-acylation site may also generate peptides without cysteine residues (thus no carbamidomethyl modification). Therefore, the results need to be cautiously interpreted. Comparing the proteomics data between control and enriched samples quantitatively can reveal potential S-acylated proteins. Alternatively, enrichment at peptide level (Alternate Protocol 1) can be performed.

Alternate Protocol 1

PRE-RESIN DIGESTION AND PEPTIDE-LEVEL ENRICHMENT

After performing the protein reduction, free cysteine blocking, and the acyl-biotin exchange procedures as described in Basic Protocol 3, the biotinylated proteins are directly digested into peptides in this protocol using an in-solution digestion method. Biotinylated peptides are then purified by the Streptavidin resin. Compared to the enrichment in protein level in Basic Protocol 3, although this protocol identifies less total potential S-acylated proteins, it allows the identification of much more specific S-acylation sites. You may choose one protocol depending on your requirement. This protocol is used specifically when identification of the specific acylated site is desired.

Materials

Methanol (Fisher, cat. A412-500)

Chloroform (Fisher, cat. C606SK-4)

2% SDS buffer (2SB buffer, see recipe)

0.5 M Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich, cat. C4706)

50 mM Tris-HCl pH 7.4 (Fisher, cat. BP152-10)

3.33 M N-Ethylmaleimide (NEM; Sigma-Aldrich, cat. 04259) in ethanol

50 mM 2,2'-Dithiodipyridine (DTDP; Sigma-Aldrich, cat. D5767) in DMF

2 M hydroxylamine (Hyd, Sigma-Aldrich, cat. 159417) pH 7.4, freshly made and keep on ice

1 mM EZ-Link™ HPDP-biotin (Thermofisher, cat. 21341)

High-capacity streptavidin agarose resin (Pierce, cat. 20357)

8M Urea (Fisher, cat. U15-500) /100mM ammonium bicarbonate (NH_4HCO_3, Sigma-Aldrich, cat. A6141), freshly prepared on the day of protein digestion

100mM NH_4HCO_3, freshly prepared on the day of protein digestion

1 M calcium chloride ($CaCl_2$, Fisher, cat. C79-3)

Trypsin (Promega, cat. V5111)

Equilibration buffer (E buffer, see recipe)

2 × Equilibration buffer (2 × E buffer, see recipe)

Washing buffer (2 M Urea, 100mM Tris pH7.4)

Elution buffer (50 mM TCEP in 100mM Tris pH7.4)

500 mM iodoacetamide (IAM, Sigma-Aldrich, cat. I1149)

Microcentrifuge tubes (Eppendorf, 2 mL, 1.5 mL)

Thermomixer (Eppendorf, cat. 5355)

Vortex mixer (Fisher Scientific, Votex-Genie 2, cat. 12-812)

3D rotator (Fisher Scientific, cat. 260100F)

Bench-top centrifuge (Fisher Scientific, accuSpin Micro 17)

Spin columns – snap cap (Pierce, cat. 69725)

C18 Tips (Pierce, 87784)

Note: To perform protein reduction, free thiol blocking, and the S-acylation sites labeling, carry out step 1 to 15 of Basic Protocol 3. Process the biotin-labeled proteins with this protocol.

Conduct the experiment at RT unless otherwise specified.

Pre-resin trypsin digestion

1. Freshly prepare 100 mM NH_4HCO_3 and 8 M Urea/100 mM NH_4HCO_3 pH8, keep at RT.
2. Re-solubilize protein pellets in 50 µL 8 M Urea/100 mM NH_4HCO_3 pH8, at 60 ºC for 30min in a Thermomixer with rotation at 1000 rpm.
3. Dilute the sample with 450 µL 100 mM NH_4HCO_3 to reduce the urea concentration to a final concentration less than 1 M. Vortex and then centrifuge at 16,000×g for 5 min to remove the un-solubilized protein aggregates.
4. Transfer the supernatant to a new centrifuge tube. Add 0.5 µL 1M $CaCl_2$ to a concentration of 1 mM $CaCl_2$.
5. Digest sample for 3 hours or overnight with Trypsin at 37ºC at a concentration of 1µg trypsin/50µg protein.

Purification of biotinylated peptides

6. Add 500 µL 2 × E buffer to achieve a final concentration of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 0.2% Triton X-100, 0.4 M urea, 50 mM NH_4HCO_3.
7. Equilibrate 100 µL volume of streptavidin-agarose resin in storage solution (50 µL bed volume) for each sample with E buffer as step 14 in Basic Protocol 3.
8. Transfer resin to samples, and incubate at RT for 60 min with rotation on a 3D rotator.
9. Transfer above solution containing streptavidin resin to a new spin column that has been placed in an original collection tube (supplied with prepacked columns). Label the column. Centrifuge at 400×g for 1 min at RT, discard flow-through.

   Note: If the solution volume is such that the entire sample cannot be added at once, centrifuge the column to allow some of the solution to pass through, discard the flow-through, and then proceed with the rest of the solution.
10. Wash resin with 600 µL E buffer for 3 times. To wash the resin thoroughly, add E buffer on top of the resin bed, press hard to close the snap cap, hold tightly to keep the column and collection tube connected to prevent leakage, and mix well by inverting 10 times.
11. Wash resin with 600 µL Washing buffer for 3 times as step 10.
12. Place the column in a new 1.5 mL centrifuge tube and centrifuge at 400 × g for 2 min. Discard the centrifuge tube.
13. Replace the bottom cap on to the column (use white tip supplied with prepacked column) and place the column into a new 1.5 mL centrifuge tube. Label the centrifuge tube.
14. To elute the enriched biotinylated peptides, add 100 µL Elution buffer to each column, incubate at 37ºC for 20min in a Thermomixer with rotation at 800 rpm, and then centrifuge tubes at 400×*g* for 2 min. Keep the flow-through.
15. Repeat step 14. Combine the flow-through.

    Note: The flow-through contains the enriched peptides with potential S-acylation sites. After thiol-acyl cleavage by hydroxylamine, biotin-labeling by HPDP-biotin, thiol-biotin capture by the streptavidin resin, and thiol-biotin cleavage by TCEP, the S-acylation sites are present as free thiols on cysteines at this time point. The sites will be further alkylated by IAM and detected by MS.

Alkylation and desalting

16. Add 4 µL 500 mM IAM solution to a final concentration of 10mM (1:50 dilution) and mix briefly. Incubate in a Thermomixer in the dark, at 25°C for 45 min with rotation at 300 rpm.
17. Use C18 clean-up tips to desalt the sample prior to MS by following the product manual.

REAGENTS AND SOLUTIONS

½ Murashige & Skoog (MS) agar medium

2.22 g/L Murashige and Skoog (MS) Basal Salt Mixture (with vitamins) (Caisson, cat. MSP09)

20 g/L sucrose

Adjust pH with 1 N KOH to 5.7

Aliquot above liquid MS medium into small conical flasks, add 0.5% phytagel (2.5 g / 500 mL) (Sigma, cat. P8169) into aliquoted medium

Autoclave for 20 min at 15 psi, 121 ℃

Cool down medium until not too hot to handle

From here on, work in the laminar flow hood

Add appreciate antibiotic into the medium, shake the medium well

Dispense medium into 100 mm X 100 mm square petri dishes, dry the medium in the laminar hood

Store plates up to 6 months at 4 ℃if no antibiotic added, store up to 1 month at 4 ℃if antibiotic added

Homogenization buffer (H buffer)

50 mM final HEPES-KOH pH 7.5 (Fisher, cat. BP310-100)

250 mM sucrose (Fisher, cat. BP220-212)

5% (v/v) glycerol (Fisher, cat. BP229-1)

10 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Sigma-Aldrich, cat. ED2SS)

0.5% (m/v) soluble polyvinylpyrrolidone (PVP-10; Sigma-Aldrich, cat. no. PVP-10)

50 mM sodium pyrophosphate (NaPP, Sigma-Aldrich, cat. P8010)

1 mM sodium molybdate dihydrate (NaMo, Sigma-Aldrich, cat. 331058)

25 mM sodium fluoride (NaF, Sigma-Aldrich, cat. S7920)

Filter-sterilize solution. Store up to 6 months at 4°C

Just prior to use, add the following per 10 mL H buffer:

100 µL 100 mM phenylmethylsulfonyl fluoride (PMSF; 1 mM final; Sigma, cat. 93842) in ethanol

10 µL 10 mM leupeptin (10 µM final; Sigma, cat. L2884)

10 µL 10 mM calyculin (resuspended in DMSO, 10 nM final; Sigma, C5552)

Proteinase inhibitor cocktail for plant tissues (e.g. cat. P 9599 from Sigma) can be used to replace the proteinase inhibitors listed in the recipe.

2% SDS buffer (2SB buffer)

50 mM Tris-HCl pH7.4 (Fisher, cat. BP152-10)

2% sodium dodecyl sulfate (SDS; Fisher, cat. BP166-500)

5 mM EDTA (Sigma-Aldrich, cat. ED2SS)

Store at room temperature.

Dilution Buffer

50 mM Tris-HCl pH 7.4 (Fisher, cat. BP152-10)

150 mM NaCl (Fisher, cat. S271-3)

5 mM EDTA (Sigma-Aldrich, cat. ED2SS)

0.2% Triton X-100 (Fisher, cat. BP151-500)

Store at room temperature.

Equilibration buffer (E buffer)

50 mM Tris-HCl pH 7.4 (Fisher, cat. BP152-10)

150 mM NaCl (Fisher, cat. S271-3)

5 mM EDTA (Sigma-Aldrich, cat. ED2SS)

0.1% SDS (Fisher, cat. BP166-500)

0.2% Triton X-100 (Fisher, cat. BP151-500)

Store at room temperature.

2 × Equilibration buffer (2 × E buffer)

100mM Tris-HCl pH 7.4 (Fisher, cat. BP152-10)

300 mM NaCl (Fisher, cat. S271-3)

10 mM EDTA (Sigma-Aldrich, cat. ED2SS)

0.2% SDS (Fisher, cat. BP166-500)

0.4% Triton X-100 (Fisher, cat. BP151-500)

Store at room temperature.

COMMENTARY

Background Information

Protein S-acylation is catalyzed by protein acyltransferases (PATs), and de-S-acylation relies on acyl-protein thioesterases (APTs) (Zheng et al., 2019). Due to the reversible characteristic, dynamic S-acylation can impact proteins' function by altering their membrane affinity in a flexible and smart manner. 24 PAT genes have been identified in *Arabidopsis* (Zheng et al., 2019). However, only *PAT4*, *PAT5*, *PAT9* and *PAT10* have been characterized and their substrates studied (Wan et al., 2017; Zhou et al., 2013; Chen et al., 2019). MtAPT1 is the only reported protein thioesterase that has been characterized in plants (Duan et al., 2017). Identification and functional characterization of the S-acylation enzymes and their substrates will elevate our understanding of S-acylation in plants.

The approaches for large-scale identification of S-acylated proteins mainly depend on proteomic methods in combination with different S-acylation site labeling and capturing methods. Three major methods have been employed to achieve proteomic profiling of S-acylated proteins: metabolic labeling followed by click chemistry (MLCC) (Kostiuk et al.,

2008), acyl-biotin exchange (ABE) (Drisdel and Green, 2004), and resin assisted capture (acyl-RAC) (Forrester et al., 2011). In MLCC, a palmitate analog is added to cell culture medium and metabolically incorporated into native S-acylation sites during culturing. After protein isolation, palmitate analog labeled proteins are conjugated to a biotin analog by click chemistry. The biotinylated proteins are then enriched by streptavidin affinity purification (Zhou et al., 2014). In ABE, free thiols are blocked by alkylating reagent (e.g., NEM), S-acylated cysteines are then hydrolyzed into free cysteines by neutral hydroxylamine. The newly exposed cysteines are conjugated to a biotin analog (e.g., HPDP-biotin), so the formerly S-acylated proteins can be enriched by streptavidin affinity purification and identified through mass spectrometry or verified by western blotting. Our protocol uses the low-background ABE (LB-ABE) method (Zhou et al., 2019), which employs additional blockage of free cysteine with 2,2′-dithiodipyridine. In the acyl-RAC strategy, a thiol-reactive resin, which can capture free thiols, replaces the biotin exchange and avidin purification steps. Using this strategy, S-acylated peptides can be more rapidly purified.

Compared to MLCC, ABE does not require metabolic labeling so it can be used to analyze S-acylated proteins in any plant tissues or cell types. In addition, ABE has the potential to capture the full S-acylproteome. A combination of plasma membrane enrichment by Brij-58 treatment and S-acylation enrichment by LB-ABE method enables us to identify more plasma membrane (PM) proteins with S-acylation.

Critical Parameters and Troubleshooting

Protein amount

Too little final protein and tryptic peptide will lead to unfavorable MS results. Almost all steps will impact the final protein levels.

- Compared to a general global proteomic analysis, much more starting plant material is needed due to two stages of protein enrichment (PM enrichment and S-acylation enrichment).
- Excessive Brij-58 in PM enrichment will result in a big loss of proteins. Using the appropriate amount of Brij-58 is crucial.
- A small amount of protein will be lost at each ultracentrifuge and precipitation step. Thus, avoid conducting unnecessary ultracentrifuge and the protein precipitation / re-solubilization procedures.
- Sufficient ABE exchange by HPDP-biotin and purification of S-acylated proteins are essential to retain the S-acylated proteins. To achieve this, an adequate amount of HPDP-biotin and Streptavidin resin are needed.

Chemical contaminants

Carry over of some reagents may impair the downstream procedures. Removing interfering contaminants is critical for these steps.

- Thiol blocking reagent NEM and DTDP will further alkylate new exposed thiol by hydroxylamine cleavage and prohibit biotin labeling. We recommend using sequential methanol/chloroform precipitation to completely remove the alkylate reagents.
- The biotinylating reagent HPDP-biotin can bind and occupy the binding sites of Streptavidin agarose resin. As a result, the Streptavidin resin capacity of capturing the S-

acylated proteins is decreased. Sequential methanol/chloroform precipitation can help remove the excess HPDP-biotin.

- Salts (e.g. NaCl) and detergents (e.g. SDS) can disrupt mass spectrometry analysis. Salts can usually be effectively removed by offline and online SPE prior to MS. Strong ionic detergent (e.g. SDS) contamination for protein solubilization is a frequent cause of failed MS analysis. SDS can be removed at the protein level using S-trap or sized based filters. But SDS is extremely difficult to remove from digested peptides. Avoid using SDS in the final buffer before desalting.

False positive and background control

False positives can be recognized as putative sites for S-acylation during the downstream analysis. Most of the false positives come from insufficient blocking of free cysteine residues. Complete blocking of original and TCEP induced free cysteines is crucial. NEM is used to primarily block the majority of the free cysteines. In addition to this, 2,2′-dithiodipyridine is employed in our protocol for further blocking. Some non-S-acylated proteins can be captured due to hydroxylamine-independent non-specific binding with biotin or resin during purification by Streptavidin resin. These co-enriched proteins will be identified as false positives.

A sample without hydroxylamine treatment (Hyd-) should be processed in parallel with the experimental sample (Hyd+) as a background control. The proteins exclusively identified from the Hyd+ samples are considered to be potential S-acylated proteins. Distinguishing S-acylated-proteins from substantial contaminant background is a big challenge. A Hyd+/ Hyd- ratio of spectral counting (SC) or label free quantitation can be referred to determine the candidates.

Understanding Results

Following the protocol of preparation of *Arabidopsis* seedlings, we can harvest 2.5 g seedlings / agar plate if grown in the growth chamber for two weeks. Four plates are combined as one sample for protein extraction. Following the protocol of enrichment of PM proteins, the final yield is ~10 - 15 mg crude membrane (CM) protein after the first ultracentrifugation step. The final enriched PM proteins (ePM) are approximately 2 - 3 mg per 8 mg starting CM. 2 mg ePM is used for enrichment of S-acylated proteins/peptides in this protocol. The rest of the ePM can be used for global proteomic or phospho-proteomic analysis.

Following the protocol of protein level enrichment (Basic Protocol 3) and proteomic analysis, we identified about 30,000 peptides in a single LC-MS/MS run, only 1% peptides contain cysteines with IAM-alkylation. Hyd+ sample identified more peptides than Hyd- sample. Using the protocol of peptide level enrichment (Alternate Protocol 1) and the proteomic analysis, thousands of peptides can be identified, over 80% of the peptides contain cysteines with IAM-alkylation. Hyd+ sample identified more peptides than Hyd- sample.

Please note, not all identified proteins in Hyd+ samples are putative S-acylated proteins. On the contrary, a large portion of proteins are non-specific background proteins. Likewise, not all IAM-alkylated cysteines are S-acylation sites. These background proteins / peptides are false positives. Comparison of spectral counting between Hyd+ and Hyd- samples enables us to remove many of the false positives. We used the following criteria for considering potential S-acylated proteins:

1) Hyd+/ Hyd- ratio is above 2;
2) At least two tryptic peptides are detected;
3) Fulfill the above two criteria in at least two replicates.

Confidence levels can be classified based on the Hyd+/ Hyd- ratio. For example, potential S-acylated proteins can be categorized into high confidence (Hyd+/ Hyd- ≥ 10), medium confidence (5 ≤ Hyd+/ Hyd- < 10), and low confidence (2 < Hyd+/ Hyd- < 5). Protein S-acylation can be validated by western blot.

Time Considerations

See Table 2 for time considerations for the protocols.

[*Insert Table 2 here]

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Literature Cited

Chen, D., Ahsan, N., Thelen, J. J., & Stacey, G. (2019). S-Acylation of plant immune receptors mediates immune signaling in plasma membrane nanodomains. *bioRxiv,* 720482. doi:10.1101/720482.

Collins, C. A., Leslie, M. E., Peck, S. C., & Heese, A. (2017b) Simplified enrichment of plasma membrane proteins from Arabidopsis thaliana seedlings using differential centrifugation and Brij-58 treatment. *Methods in Molecular Biology*, 1564, 155–168.

Collins, M. O., Woodley, K. T., & Choudhary, J. S. (2017a). Global, site-specific analysis of neuronal protein S-acylation. *Scientific Report,* 7, 4683. doi: 10.1038/s41598-017-04580-1.

Drisdel, R. C., & Green, W. N. (2004). Labeling and quantifying sites of protein palmitoylation. Biotechniques, 36, 276–285.

Duan, M., Zhang, R., Zhu, F., Zhang, Z., Gou, L., Wen, J., … Wang, T. (2017) A lipid-anchored NAC transcription factor is translocated into the nucleus and activates Glyoxalase I expression during drought stress. *Plant Cell*, 29, 1748–1772.

Fic, E., Kedracka-Krok, S., Jankowska, U., Pirog, A., & Dziedzicka-Wasylewska, M. (2010). Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis*, 31, 3573–3579.

Forrester, M.T., Hess, D.T., Thompson, J.W., Hultman, R., Moseley, M.A., Stamler, J. S., & Casey, P. J. (2011). Site-specific analysis of protein S-acylation by resin-assisted capture. *Journal of Lipid Research*, 52, 393–398.

Hemsley, P. A., Weimar, T., Lilley, K. S., Dupree, P., & Grierson, C. S. (2013). A proteomic approach identifies many novel palmitoylated proteins in *Arabidopsis*. *New Phytologist,* 197, 805–814.

Kostiuk, M. A., Corvi, M. M., Keller, B. O., Plummer, G., Prescher, J. A., Hangauer, M. … Berthiaume, L. G. (2008). Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *The FASEB Journal*. 22, 721–732.

Li, Y., & Qi, B. (2017). Progress toward understanding protein S-acylation: prospective in plants *Frontiers in Plant Science*, 8, p. 346

Turnbull, D., & Hemsley, P.A. (2017). Fats and function: protein lipid modifications in plant cell signalling. Current Opinion in Plant Biology, 40, 63–70.

Wan, Z. Y., Chai, S., Ge, F. R., Feng, Q. N., Zhang, Y., & Li, S. (2017). Arabidopsis protein S-acyl transferase 4 mediates root hair growth. *Plant Signaling and Behavior*, 90, 249–260.

Yang, W., Di Vizio D., Kirchner, M., Steen, H., & Freeman, M. R. (2010). Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Molecular & Cellular Proteomics*. 9, 54–70.

Zheng, L., Liu, P., Liu, Q., Wang, T., & Dong, J. (2019). Dynamic Protein S-Acylation in Plants. *International Journal of Molecular Sciences*, 20, 560. doi: 10.3390/ijms20030560.

Zhou, B., An, M., Freeman, M. R., & Yang, W. (2014). Technologies and Challenges in Proteomic Analysis of Protein S-acylation. *Journal of Proteomics and Bioinformatics*, 7, 256–263.

Zhou, B., Wang, Y., Yan, Y., Mariscal, J., Di Vizio, D., Freeman, M., & Yang, W. (2019). Low-background Acyl-biotinyl exchange largely eliminates the co-isolation of non-S-Acylated proteins and enables deep S-Acylproteomic analysis. *Analytical Chemistry*, 91, 9858–9866.

Zhou, L. Z., Li, S., Feng, Q. N., Zhang, Y. L., Zhao, X., Zeng, Y. L., … Zhang, Y. (2013) Protein S-acyltransferase10 is critical for development and salt tolerance in *Arabidopsis*. *Plant Cell*, 25, 1093–1107.

FIGURE LEGENDS:

Figure 1. Workflow for purification and identification S-acylated proteins in *Arabidopsis* seedlings. *Arabidopsis* seeds are germinated and grown on ½ MS agar plates in a growth chamber and harvested after treatment with required conditions (Basic Protocol 1). Membrane proteins are isolated and enriched using a simplified method involving Brij-58 treatment and ultracentrifugation (Basic Protocol 2). Proteins with S-acylation are reduced by TCEP, alkylated by NEM and 2,2'-Dithiodipyridine, and subsequently enriched by Acyl-Biotin Exchange (ABE) and streptavidin resin purification (Basic Protocol 3). Enriched proteins are trypsin-digested and identified by global mass spectrometry (Basic Protocol 4).

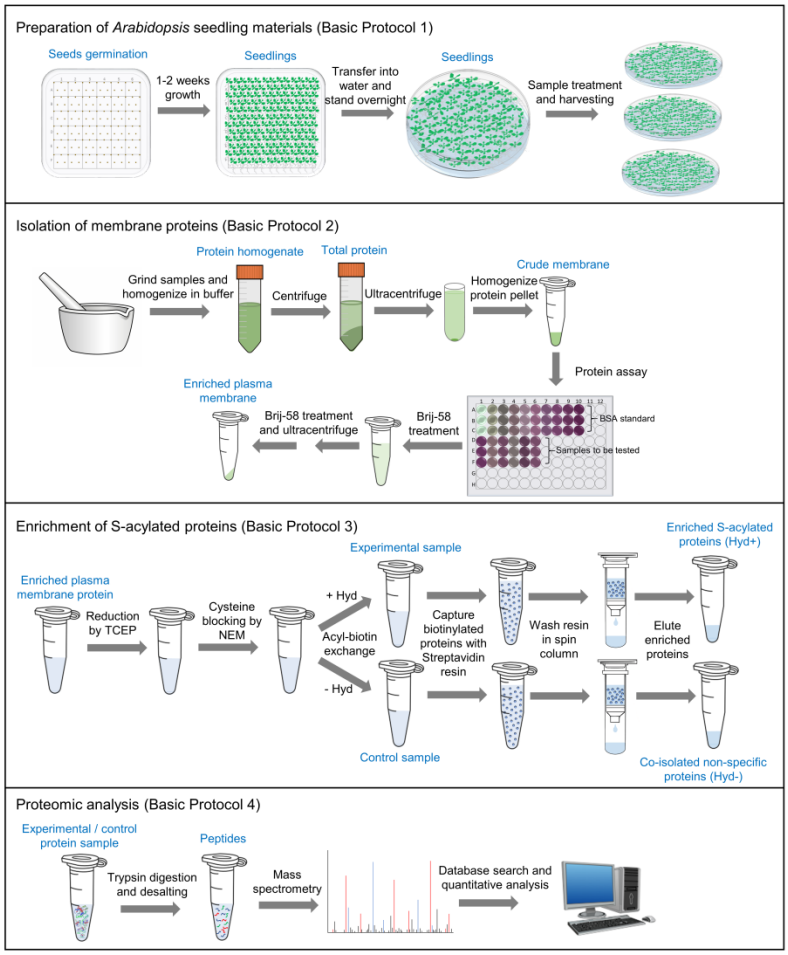
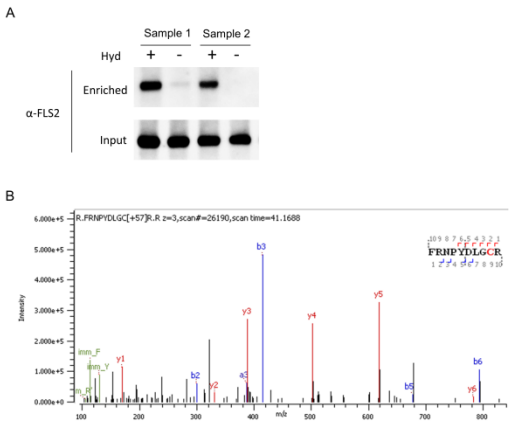


Figure 2. Enrichment and identification of S-acylated proteins using mass spectrometry. (A) Successful enrichment of the known S-acylated protein AtFLS2 (AT5G46330) in Hyd+ samples shown by western blotting. Hyd+, hydroxylamine present; Hyd-, hydroxylamine absent. (B) Representative MS/MS spectrum of the S-acylated Cysteine peptide $^{\#}$FRNPYDLGCR$^{\#}$ from AtTIP1 (AT5G20350, a known protein acyltransferase) with IAM-labeling on Cysteine.



TABLES

Table 1. Representative gradient of MPB for peptide separation.

Time (min)	% MPB
0	1
2	8
20	12
75	30
97	45
100	95
110	95
115	1
150	1

Table 2. Time considerations for each protocol

Protocol	Section	Time
Basic Protocol 1 Preparation of *Arabidopsis* seedling materials	Seed sterilization and cold treatment	3 day
	Seed germination and seedling growth	7 - 14 day
Basic Protocol 2 Isolation and enrichment of membrane proteins	Isolation of crude membrane	8 hr
	Enrichment of PM protein	8 hr
Basic Protocol 3 Enrichment of S-acylated proteins	Protein reduction and free thiol blocking	10 hr
	Acyl-Biotin Exchange	3 hr
	Purification of biotinylated proteins	5 hr
Basic Protocol 4 Trypsin digestion and proteomic analysis	Alkylation and trypsin digestion	4 hr
	Mass spectra data acquisition	3 hr
	Database searching	1 hr
Alternate Protocol 1 Pre-resin digestion and peptide level enrichment	Pre-resin trypsin digestion	4 to 12 hr
	Purification of biotinylated peptides	5 hr
	Alkylation and desalting	2 hr