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Protein Isolation from Plasma Membrane, Digestion and Processing for Strong Cation Exchange Fractionation

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[Abstract] Plasma membrane (PM) proteins play crucial roles in diverse biological processes. But their low abundance, alkalinity and hydrophobicity make their isolation a difficult task. This protocol describes an efficient method for PM proteins isolation, digestion and fractionation so that they can be well prepared for mass spectrometry analysis.
Keywords: Plasma membrane proteins, *Rapi*Gest SF, Trypsin, Strong cation exchange, Fractionation

[Background] Plasma membrane (PM) proteins participate in diverse biological processes including signal transduction, ion transport and membrane trafficking, and are the first responders in cell-environment communication. They have a complicated composition varying from species, cell types and developmental stages (Alexandersson *et al.*, 2008). Revealing their components and the expression features comprehensively with mass spectrometry (MS) is of great importance for developmental biology. However, their hydrophobic nature and the low abundance are a big challenge for the proteomic analysis (Wu and Yates, 2003). Additives like normal surfactants, organic solvents and urea are often used to improve PM proteins' solubility, but they will reduce the proteases' activities and create ion suppression during MS analysis (Zhang, 2015). *Rapi*Gest SF is a novel acid-labile anionic surfactant, which is structurally and functionally similar to SDS but does not inhibit the common endopeptidases activities at low concentration (0.1% w/v). Thus *Rapi*Gest SF used in solubilizing PM proteins can not only facilitate their digestion by exposing cleavage sites but is also easily quenched by strong acid and removed through centrifugation so that the surfactant does not affect the MS identification (Yu *et al.*, 2003). Peptides yield by the *Rapi*Gest SF-assisted digestion can directly undergo the strong cation exchange (SCX) fractionation (Yang and Wang, 2017), so that the low abundance peptides can be detected by the MS.

Materials and Reagents

1. 1.5 ml tubes (Corning, Axygen®, catalog number: MCT-150-C)
2. ZipTip® pipette tip (Merck Millipore, catalog number: ZTC18S096)
3. Pierce™ Spin columns, screw cap (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 69705)
4. C18 (FUJIGEL HANBAI, catalog number: MB 100 - 40/75)

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5. Tris (2-carboxyethyl) phosphine hydrochloride solution, 0.5 M, pH 7.0 (TCEP) (Sigma-Aldrich, catalog number: 646547)
6. Formic acid (Sigma-Aldrich, catalog number: 94318)
7. Acetonitrile (Sigma-Aldrich, catalog number: 34851)
8. *Rapi*Gest SF surfactant (WATERS, catalog number: 186001861)
9. Iodoacetamide (Sigma-Aldrich, catalog number: I1149)
10. Trypsin (Roche Diagnositics, catalog number: 11418025001)
11. Potassium phosphate dibasic trihydrate ($K_2HPO_4{\cdot}3H_2O$)
12. Potassium phosphate monobasic (KH_2PO_4)
13. Phosphoric acid (H_3PO_4)
14. Ammonium chloride (Sigma-Aldrich, catalog number: A9434)
15. Hydrochloric acid (HCl)
16. 0.5 M iodoacetamide stock (see Recipes)
17. 0.1 µg/µl trypsin stock (see Recipes)
18. 50 mM potassium phosphate buffer, pH 7.8 (see Recipes)
19. 100 ml solvent A (5 mM NH_4Cl, 25% [v/v] acetonitrile, pH 3.0) (see Recipes)
20. 100 ml solvent B (500 mM NH_4Cl, 25% [v/v] acetonitrile, pH 3.0) (see Recipes)

Equipment

1. 2 µl, 10 µl, 100 µl, 1,000 µl Pipetman (Gilson, France)
2. Optimal MAX-XP ultracentrifuge (Beckman Coulter, model: Optimal MAX-XP)
3. AKTA purifier-10 (GE Healthcare, model: AKTApurifier 10)
4. ISS 110 SpeedVac System (Thermo Fisher Scientific, Thermo Scientific™, model: ISS 110)
5. PolySULFOETHYL A™ columns, 2.1 x 200 mm, 5 µm particles, 300 Å poresize (PolyLC, catalog number: 202SE05)

Software

1. UNICORN 5.2 software (GE Healthcare, USA)

Procedure

A. Plasma membrane protein preparation
   1. Suspend the plasma membrane (PM) pellets (isolated according to Han *et al.*, 2010) with 100 µl 50 mM potassium phosphate buffer, pH 7.8.
      Note: The recommended PM protein concentration is 1 µg/µl. This protocol set 100 µg PM protein as an example, so use 100 µl buffer to suspend the pellets.

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2. Add 25 µl 1% (w/v) *Rapi*Gest SF stock to the working concentration of 0.2% (w/v), mix well and then boil the mixture at ~100 °C for 5 min to dissolve proteins from PM.
   Note: RapiGest SF is an acid labile denaturant (Figure 1A) which could solubilize and unfold proteins to make them more amenable for cleavage (Figure 1B). The recommended RapiGest SF working concentration is 0.1% (w/v). For PM proteins increase the incubation time (at 37 °C, up to 1 h is safe; at 100 °C, 5 min is enough) or use higher RapiGest SF concentration (up to 0.5% [w/v] is safe) could make the following digestion more efficient.

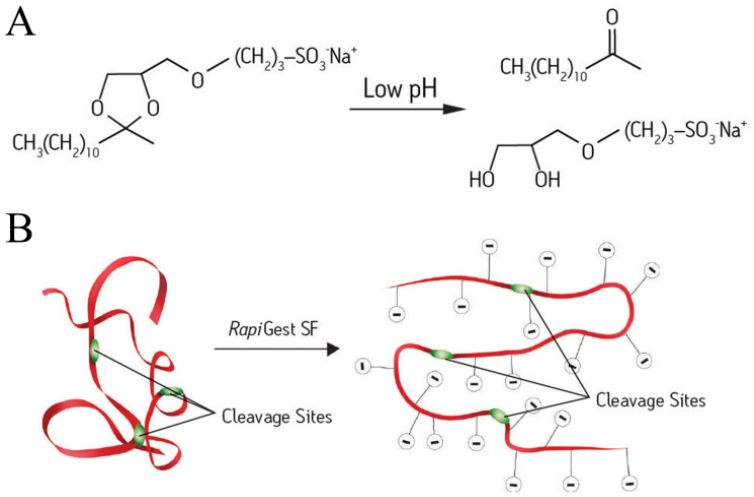

Figure 1. Working principle of *Rapi*Gest SF surfactant. A. *Rapi*Gest SF can be easily broken down by acidification and then removed by centrifugation. B. *Rapi*Gest SF is a mild denaturant which making proteins more amenable for cleavage without disrupting the endoproteases activity. Pictures were downloaded from the official website of Waters: (http://www.waters.com/waters/en_US/RapiGestSFSurfactant/nav.htm?locale=/&cid=1000941)

3. Cool down the mixture to room temperature (RT) for the following digestion.

B. Plasma membrane protein digestion
   1. Reduction: add 2.5 µl 0.5 M TCEP, pH 7.0 to the protein mixture to a working concentration of 10 mM, mix them gently and then incubate at 56 °C for 1 h.
   2. Alkylation: cool down the mixture to RT, and add 14 µl 0.5 M iodoacetamide stock to a working concentration of 50 mM, mix them gently and then incubate at RT, dark, for 45 min.
   3. Digestion: add 20 µl 0.1 µg/µl trypsin stock to the protein mixture to a working concentration of 1:50 (w/w), mix them gently and then incubate at 37 °C for 12 h.
   4. Acidification: add 0.5 µl formic acid to the mixture to a working concentration of 0.2-0.5% (v/v) (pH < 2), incubate at 37 °C for 30-45 min, then centrifuge at 4 °C, 20,000 *x g* for 20 min to remove *Rapi*Gest SF. Discharge the pellets, transfer the supernatant to a new tube.
   5. Desalting: use C18 filled spin column to concentrate and purify the peptides mixture.

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Note: If the peptides mixture is less than 5.0 µg, please use ZipTip® pipette tip to do the desalting.

6. Put the purified peptides mixture into SpeedVac system to lyophilize for the following fractionation use.

C. Peptides mixture fractionation

The following protocol was modified from Zhu *et al.* (2009). It performs on the AKTA purifier-10 system with the strong cation exchange (SCX) PolySULFOETHYL A™ column.

1. Resuspend the lyophilized peptides mixture in solvent A.
2. Run solvent B for 20 min, then solvent A for 30 min through the AKTA purifier-10 system to balance the PolySULFOETHYL A™ column.
3. Load the suspended peptides and run the following gradient at a flow rate of 200 µl/min, collect the products in every 2 min.
   0-5 min: 100% solvent A
   5-95 min: 0-60% solvent B
   95-115 min: 60%-100% solvent B
   115-145 min: 100% solvent B
   145-155 min: 100% solvent A
4. Run ddH_2O for 20 min, then 100% acetonitrile for 10 min to store the column.
5. Pool the products according to the 214 nm UV absorption peak (Figure 2).

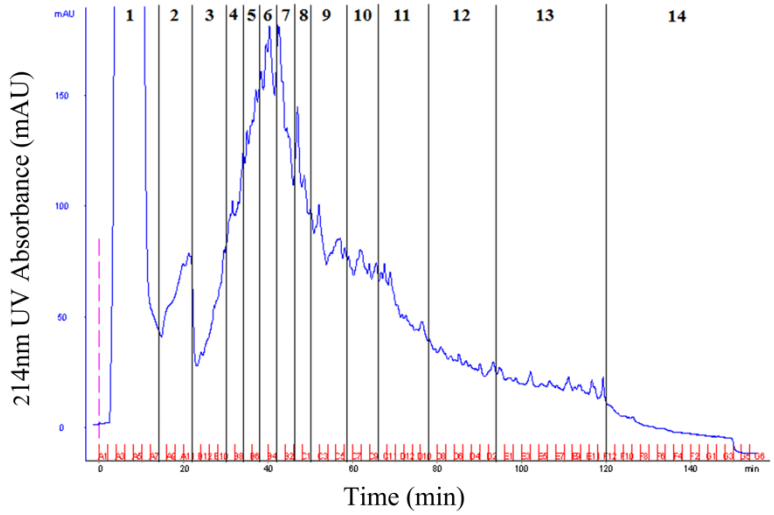

Figure 2. SCX fractionation of peptides mixture from rice mature pollen grain. According to the 214 nm UV absorption peak, the peptides mixture can be pooled into 14 fractions for LC-MS/MS analysis.

6. Use C18 spin column or ZipTip® pipette tip to purify the combined products.
7. Lyophilize the purified products for mass spectrometry identification.

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Data analysis

The SCX separated peptides were monitored by their 214 nm UV absorption peak through the UNICORN 5.2 software (GE Healthcare, USA).

Notes

After SCX fractionation, the repooled peptides contains a high concentration of salts. Don't forget to do the desalting procedure through C18 spin column or ZipTip® pipette tip, or the following mass spectrometry identification will be affected.

Recipes

1. 1% (w/v) *Rapi*Gest SF stock
   Dissolve 1 mg of lyophilized *Rapi*Gest SF powder in 100 µl ddH_2O, store at -20 °C
2. 0.5 M iodoacetamide stock
   Dissolve 46 mg of iodoacetamide powder in 500 µl ddH_2O, use immediately
3. 0.1 µg/µl trypsin stock
   Dissolve 25 µg of trypsin powder in 250 µl ddH_2O, store at -20 °C
4. 200 ml 50 mM potassium phosphate buffer, pH 7.8
   2.072 g $K_2HPO_4 \cdot 3H_2O$
   0.125 g KH_2PO_4
   Fill up to 200 ml with ddH_2O
   Adjust pH to 7.8 with H_3PO_4
5. 100 ml solvent A (5 mM NH_4Cl, 25% [v/v] acetonitrile, pH 3.0)
   0.027 g NH_4Cl
   25 ml acetonitrile
   Fill up to 100 ml with ddH_2O
   Adjust pH to 3.0 with HCl
6. 100 ml solvent B (500 mM NH_4Cl, 25% [v/v] acetonitrile, pH 3.0)
   2.675 g NH_4Cl
   25 ml acetonitrile
   Fill up to 100 ml with ddH_2O
   Adjust pH to 3.0 with HCl

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References

1. Alexandersson, E., Gustavsson, N., Bernfur, K., Karlsson, A., Kjellbom, P. and Larsson, C. (2008). Purification and proteomic analysis of plant plasma membranes. *Methods Mol Biol* 432: 161-173.
2. Han, B., Chen, S., Dai, S., Yang, N. and Wang, T. (2010). Isobaric tags for relative and absolute quantification- based comparative proteomics reveals the features of plasma membrane-associated proteomes of pollen grains and pollen tubes from *Lilium davidii*. *J Integr Plant Biol* 52(12): 1043-1058.
3. Wu, C. C. and Yates, J. R., 3rd (2003). The application of mass spectrometry to membrane proteomics. *Nat Biotechnol* 21(3): 262-267.
4. Yang, N. and Wang, T. (2017). Comparative proteomic analysis reveals a dynamic pollen plasma membrane protein map and the membrane landscape of receptor-like kinases and transporters important for pollen tube growth and interaction with pistils in rice. *BMC Plant Biol* 17(1): 2.
5. Yu, Y. Q., Gilar, M., Lee, P. J., Bouvier, E. S. and Gebler, J. C. (2003). Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. *Anal Chem* 75(21): 6023-6028.
6. Zhang, X. (2015). Less is more: Membrane protein digestion beyond urea-trypsin solution for next-level proteomics. *Mol Cell Proteomics* 14(9): 2441-2453.
7. Zhu, M., Dai, S., McClung, S., Yan, X. and Chen, S. (2009). Functional differentiation of *Brassica napus* guard cells and mesophyll cells revealed by comparative proteomics. *Mol Cell Proteomics* 8(4): 752-766.