Proteolytic Fragment Isolation and Analysis (ex. N-terminal GST-tagged CLAVATA3 Protein GST-CLV3)

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[Abstract] It has become clear that the post-embryonic growth and development of plants requires properly controlled short distance cell-to-cell communication not only through the historically well-known phytohormones, but also through secreted small peptide signals. This protocol demonstrates an example of how to isolate small peptides (< 10 daltons) from complex protein mixtures (*e.g.* cauliflower meristem protein extraction) for MS/MS analysis.

Materials and Reagents

1. Glutathione Sepharose 4B (Amersham biosciences , catalog number: 17-0756-01)
2. Glutathione (Thermo scientific, catalog number: 78259)
3. Protease inhibitor cocktail (Sigma-Aldrich, catalog number: P9599)
4. Triton X-100 (Pierce, catalog number: 85111)
5. GST-mCLV3 proteins
6. Tris-HCl
7. Hepes (Sigma-Aldrich, catalog number: H3375)
8. EDTA (Sigma-Aldrich, catalog number: ED100g)
9. Phenylmethylsulfonyl fluoride (Sigma-Aldrich, catalog number: 78830-1G)
10. Aprotinin (Sigma-Aldrich, catalog number: A4529-1MG)
11. Chymostatin (Sigma-Aldrich, catalog number: C7268-1MG)
12. Leupeptin (Sigma-Aldrich, catalog number: L2884-5MG)
13. Eluting buffer (see Recipes)
14. Cauliflower extraction buffer (see Recipes)

Equipment

1. Rotor
2. Microcon YM-10 centrifugal filter (EMD Millipore)
3. Tabletop centrifuge

4. Mass spectrometer (4700 proteomics analyzer)

Procedure

A. Cauliflower (*Brassica oleracea*) meristem protein extracts were prepared as described before (Trotochaud *et al.*, 1999) with or without 0.1% Triton X-100. Note that 1 ml of protease inhibitor cocktail for use with plant cell extracts was added in the extraction buffer per 300 *x g* of tissues. Before use, the extracts were centrifuged at 40,000 *x g* for 30 min at 4 °C. It is very important to use fresh cauliflower. We usually try to get cauliflower from local farms. The meristem tissues were collected using razor blade "shaving" the head of the cauliflower.

B. Incubate ~100 µg purified GST-mCLV3 proteins (mature CLV3 protrein) with ~400 µl cauliflower protein extracts for 2 h at room temperature (RT) on a rotor.

C. For N-terminal tagged fragment (~ 32-34 kD) isolation.
   1. Bound protein mixtures to Glutathione Sepharose 4B.
   2. Elute the N-terminal GST containing fragments from the beads with buffer containing 10 mM Glutathione, 50 mM Tris-HCl (pH 8.0).
   3. If needed, concentrate protein elution using Microcon YM-50 centrifugal filter at 14,000 *x g*, 4 °C for 30 min and elute in desired volume and buffer (*e.g.* 0.1% TFA trifluoroacetic acid/water) for MS analysis.

D. For C-terminal untagged fragment (~ 2-4 kD) isolation.
   1. Subject protein mixtures to centrifuge through Microcon YM-10 centrifugal filter at 14,000 *x g*, 4 °C for 30 min.
   2. Collect the flow-through fractions for intact MS analysis.
   3. Further characterize peaks of interest by MS/MS analysis (control: Cauliflower protein extracts only; GST-CLV3 incubated with cauliflower extraction buffer).

Recipes

1. Eluting buffer
   50 mM Tris-HCl
   10 mM reduced glutathione (pH 8.0)
2. Cauliflower extraction buffer
   50 mM Hepes (pH 7.4)

10 mM EDTA
0.1% Triton X-100
1 mM phenylmethylsulfonyl fluoride
5 mg/ml aprotinin
10 mg/ml chymostatin
1 mg/ml leupeptin
For extracts without membrane proteins, exclude Triton X-100 from the buffer.

Acknowledgments

This protocol has been adapted from previous publications including Ni and Clark (2006), Ni *et al.* (2011) and Trotochaud *et al.* (1999).

References

1. Ni, J. and Clark, S. E. (2006). Evidence for functional conservation, sufficiency, and proteolytic processing of the CLAVATA3 CLE domain. *Plant Physiol* 140(2): 726-733.
2. Ni, J., Guo, Y., Jin, H., Hartsell, J. and Clark, S. E. (2011). Characterization of a CLE processing activity. *Plant Mol Biol* 75(1-2): 67-75.
3. Trotochaud, A. E., Hao, T., Wu, G., Yang, Z. and Clark, S. E. (1999). The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* 11(3): 393-406.

