

**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 protein) 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.