Chapter 5

Purification of Plant Receptor Kinases from Plant Plasma Membranes

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Abstract

Receptor kinases play a central role in various biological processes, but due to their low abundance and highly hydrophobic and dynamic nature, only a few of them have been functionally characterized, and their partners and ligands remain unidentified. Receptor protein extraction and purification from plant tissues is one of the most challenging steps for the success of various biochemical analyses to characterize their function. Immunoprecipitation is a widely used and selective method for enriching or purifying a specific protein. Here we describe two different optimized protein purification protocols, batch and on-chip immunoprecipitation, which efficiently isolate plant membrane receptor kinases for functional analysis.

Key words Receptor kinase, Purification, Protein-protein interaction, Immunoprecipitation, Biosensor chip

1 Introduction

The plasma membrane is a complex structure that separates the cell from its external environment while providing a means for cellular communication. Membrane-bound receptor kinases are composed of an ectodomain generally involved in ligand binding, a single pass transmembrane domain, and an intracellular kinase domain. There are more than six hundred receptor kinases encoded by the *Arabidopsis* genome which coordinate extracellular signals to regulate numerous biological processes including growth, development, and defense [1–7]. However, despite their importance in biology, it is not fully understood how receptor kinases form a signaling complex at the plasma membrane. Furthermore, among a handful of functionally characterized receptor kinases, their partners and ligands remain unidentified. Their low natural abundance, instability in nonnative environments, and highly hydrophobic and dynamic nature are factors that have complicated the research process and contributed to the lack of understanding. The functional study of receptor kinases starts with protein extraction and

Reidunn Birgitta Aalen (ed.), *Plant Receptor Kinases: Methods and Protocols*, Methods in Molecular Biology, vol. 1621,
DOI 10.1007/978-1-4939-7063-6_5,

purification from a complex mixture (such as cell lysates) followed by various biochemical and proteomics studies. To be able to conduct functional analysis, it is therefore important to use a proper method to enrich or purify a protein of interest. Immunoprecipitation (IP) is a powerful separation technique, widely used to purify a protein [8]. However, because of the complexities associated with studying a membrane receptor kinase *in planta*, there are several variables and factors that should be considered when designing an IP experiment to isolate adequate amounts and successfully purify this protein.

The ERECTA-family leucine-rich repeat (LRR) receptor kinases, ERECTA, ERL1, and ERL2, control a wide range of biological processes, including organ growth, reproductive tissue development, immunity, and stomatal patterning [4, 9, 10]. Several *EPIDERMAL PATTERNING FACTOR* (*EPF*) family members that encode small cysteine-rich peptides have recently been discovered as signals for the ERECTA-family to control two different biological processes: stomata development and aboveground growth [11–15]. The methods described below were used in studying ligand-receptor and receptor-receptor interactions, as well as their binding affinity. Co-IP assays using solubilized microsomal fractions, which were isolated from double-transgenic *Arabidopsis* plants expressing pairwise combinations of epitope-tagged receptors, demonstrated that the ERECTA-family forms receptor homodimers as well as heterodimers with the LRR protein TOO MANY MOUTHS (TMM), while TMM does not homodimerize in vivo [12]. In addition to these receptor-receptor interactions, Co-IP or IP of ERECTA-GFP from purified membrane fractions, followed by biochemical studies, was used to detect associations of EPF ligands and ERECTA receptor kinase [11–13]. Furthermore, identification of EPF ligands as well as optimized receptor purification methods successfully provided the experimental setups to discover that two contrasting peptides, EPF2 and STOMAGEN/EPFL9, compete for binding to the same receptor kinase, ERECTA, to fine-tune stomatal patterning [13]. Here we describe two different protein purification protocols, batch and on-chip IP, from plants either stably or transiently expressing tagged proteins, to prepare membrane receptor kinases efficiently for various biochemical analyses in order to study their function. These methods should also be readily applicable to other membrane receptor kinases in plants.

2 Materials

2.1 Purification of Microsomal Membrane Fractions

1. Mortars and pestles.
2. Sonicator.
3. Empty chromatography columns (731-1550, Bio-Rad).

4. Ultracentrifuge capable of 100,000 × *g* at 4 °C.
5. Homogenization buffer: 100 mM Tris–HCl pH 8.8, 150 mM NaCl, 1 mM EDTA, 20% glycerol, 0.75% polyvinylpolypyrrolidone (PVPP), 20 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and 1 complete protease inhibitor cocktail (Roche) per 50 mL extraction buffer.

   Make buffers fresh using premade stock solutions.

   Add solid insoluble PVPP to the extraction buffer 1–2 h before use; allow hydrating fully (*see* **Note 1**).
6. 100 mM phenylmethanesulfonyl fluoride (PMSF) in isopropanol.
7. YEB medium: beef extract (5 g/L), yeast extract (1 g/L), peptone (5 g/L), sucrose (5 g/L), and $MgSO_4 \cdot 7H_2O$ (0.5 g/L), pH 7.0.
8. Infiltration medium: 10 mM $MgCl_2$, 10 mM MES (pH 5.6), and 150 μM acetosyringone.

   Make buffers fresh using premade stock solutions.

2.2 Membrane Protein Extraction

1. Sonicator.
2. Membrane solubilization buffer: 100 mM Tris–HCl at pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM PMSF, and 1 complete protease inhibitor cocktail (Roche) per 50 mL extraction buffer.
3. Ultracentrifuge capable of 100,000 × *g* at 4 °C.
4. Bradford Protein Assay Kit (Bio-Rad).

2.3 Immunoprecipitation

1. Magnet separator (Invitrogen).
2. Mixer allowing tilting and rotation of tubes.
3. Dynabeads Protein G (Invitrogen).
4. Anti-GFP (ab290, Abcam).
5. Phosphate buffer saline (PBS) pH 7.4 with and without 0.1% Tween 20.

2.4 On-chip Receptor Protein Purification

1. Biosensor chip.
2. UV plasma.
3. Plate shaker.
4. Anti-GFP (ab290, Abcam).
5. PBS, pH 7.4.
6. PBS with 2% bovine serum albumin (BSA).

3 Methods

3.1 Preparing Plant Material

Arabidopsis Seedlings

1. *Arabidopsis* seedlings, expressing full-length receptor with an epitope tag, are obtained by sterilizing seeds in 33% bleach solution with 0.1% Triton for 10–12 min, followed by extensive washing with sterile water (*see* **Note 2**).
2. Sow seeds on ½ MS 1% sucrose agar plates and keep in a cold room in the dark for 2–3 days.
3. Grow them for 12 days in a regular growth room (16 h light/8 h dark cycle, temperature of 22 °C) and freeze samples in liquid nitrogen (*see* **Note 3**).
4. Grind frozen tissue to fine powder with prechilled mortar and pestle in liquid nitrogen.
5. Immediately transfer the finely ground powder into two 1.5 mL microcentrifuge tubes precooled in liquid nitrogen. The volume of the powder in each 1.5 mL tube must be around 0.5 mL.
6. The powder sample can be immediately used or stored in a −80 °C freezer until protein extraction (*see* **Note 4**).

Nicotiana benthamiana Leaves

1. Inoculate single colony of *Agrobacterium* that has been previously transformed with the epitope-tagged receptor and silencing suppressor p19 expression clone in 3 mL LB with appropriate antibiotics, respectively. Grow overnight at 28 °C, 200 rpm.
2. Use 1 mL of the overnight culture to inoculate 20 mL YEB medium with same antibiotics, plus 150 μM acetosyringone and 10 mM MES (pH 5.6) (*see* **Note 5**) and grow overnight at 28 °C, 200 rpm.
3. Precipitate the bacteria culture (5000 × *g*, 15 min at room temperature) and resuspend the pellet in 3 mL of the infiltration medium (*see* **Note 6**).
4. Measure the absorbance at 600 nm of a 1 in 10 dilution of resuspended culture and adjust the final OD_{600} to 1 with infiltration medium.
5. Leave on the bench at room temperature for 4–5 h before infiltration.
6. Just before infiltration, mix *Agrobacterium* suspensions that allow expression of p19 and receptor to have an optical density at 600 nm of 0.3 for each strain.
7. The mixed bacterial suspensions were then infiltrated into young, but fully expanded, leaves of tobacco plants using a needleless 1 mL syringe (*see* **Note 7**).

8. After infiltration, incubate plants under normal growing conditions (16 h light/8 h dark cycle, temperature of 22 °C) for 2 days to generate transgenic tobacco plants transiently expressing tagged receptor kinase (*see* **Note 8**) and freeze leaf tissues within the infiltrated zones in liquid nitrogen.
9. Grind frozen tissue to fine powder with prechilled mortar and pestle in liquid nitrogen.
10. Immediately transfer the powder into 50 mL tubes precooled in liquid nitrogen. The volume of the powder in each 50 mL tube must be around 5 mL.
11. Store the powder sample in a −80 °C freezer until protein extraction.

3.2 Purification of Microsomal Membrane Fraction

Arabidopsis Microsomal Membrane Fraction

1. Prepare fresh extraction buffer with presoaked 0.75% PVPP to remove phenolic compounds (1 mL per 0.5 mL volume of the tissue powder).
2. Add two volumes of cold homogenization buffer to the tube with ground powder and vortex immediately so that all the ground powder is quickly mixed with extraction buffer. The tissue should never be allowed to thaw.
3. Incubate at 4 °C for 10 min. with gentle mixing.
4. Centrifuge lysate at 10,000 × *g* at 4 °C for 10 min. This step may be repeated if necessary.
5. Transfer the supernatant to a new tube to remove cell debris and PVPP.
6. Perform ultracentrifugation at 100,000 × *g* for 30 min at 4 °C.
7. Remove the supernatant containing soluble proteins. The compact pellet obtained contains all of the cell's microsomal fraction.

Tobacco Microsomal Membrane Fractions

1. Prepare fresh extraction buffer with presoaked 0.75% PVPP to remove phenolic compounds (15 mL per 5 mL volume of the tissue powder).
2. Add three volumes of cold homogenization buffer to the tube with ground powder and vortex immediately so that all the ground powder is quickly mixed with extraction buffer. The tissue should never be allowed to thaw to prevent degradation.
3. Incubate at 4 °C for 10 min. with gentle mixing.
4. Centrifuge lysate at 10,000 × *g* at 4 °C for 10 min.
5. Transfer the supernatant to a new tube and then pass the supernatant through an empty column (Bio-Rad) to trap cell debris and PVPP (*see* **Note 9**).
6. Sonicate the flow through using two 10-s pulses (30 s in between pulses) using a probe sonicator (*see* **Note 10**).

7. Perform ultracentrifugaton at 100,000 × *g* for 30 min at 4 °C.
8. Remove the supernatant containing soluble proteins. The compact pellet obtained contains all of the cell's microsomal fraction.

3.3 Membrane Protein Extraction

1. Add 1 mL membrane solubilization buffer for 1 g of *Arabidopsis* samples and 2 ml membrane solubilization buffer for 5 g of tobacco samples and then resuspend well to bring the pellet into solution.
2. Sonicate the sample using two 10s pulses (30 s in between pulses) using a probe sonicator and allow the extract to incubate with the buffer for at least 30 min with gentle mixing at 4 °C.
3. Perform ultracentrifugation at 100,000 × *g* for 30 min at 4 °C to remove any insoluble particles.
4. Transfer the supernatant to a fresh tube and measure the protein amount using the Bradfrod assay kit.
5. Adjust and transfer 2–2.5 mg/mL protein to a new tube and keep on ice in the cold room for future use. Take a small aliquot to use as input and mix with 4× sample buffer.

3.4 Immunoprecipitation

1. Resuspend Dynabeads Protein G (Invitrogen) in the vial with rotation for 5 min.
2. Transfer resuspended 25 μL Dynabeads Protein G to a 1.5 mL microcentrifuge tube.
3. Place the tube on the magnet stand to separate the beads from the solution, and remove the supernatant.
4. Resuspend with 200 μL PBS by gentle pipetting.
5. Place the tube on the magnet stand to separate the beads from the solution, and remove the supernatant.
6. Remove the tube from the magnet stand and add 1 μL anti-GFP (Abcam) diluted in 200 μL PBS with Tween 20.
7. Incubate with rotation overnight at 4 °C.
8. The next day, place the tube on the magnetic stand and remove the supernatant.
9. Resuspend with 200 μL PBS with Tween 20 by gentle pipetting twice.
10. Add 2–2.5 mg/mL protein extract from Subheading 3.3, **step 5** to the tube and incubate with rotation for 1–2 h at 4 °C (*see* **Note 11**).
11. Place the tube on the magnet stand to separate the beads from the solution, and remove the supernatant.
12. Wash at least 3–4 times using 500 μL PBS without or with Tween 20 for each wash.

13. Resuspend with 200 μL PBS and transfer the bead suspension to a clean 1.5 mL tube. This is recommended to avoid co-elution of proteins bound to the tube wall.
14. Place the tube on the magnet stand to separate the beads from the solution, and remove the supernatant.
15. At this point, the receptor kinase is purified by IP and can be used for interaction studies with other proteins such as peptide ligands (*see* **Note 12**).

3.5 On-chip Receptor Protein Purification

1. Place the biosensor chip into 1:1 methanol/acetone solution and sonicate the chip in methanol:acetone mixture for 5 min in sonication bath.
2. Take out the biosensor chip from methanol:acetone mixture and remove the liquid from the surface of the chip by blotting the chip in paper Kim wipes (*see* **Note 13**).
3. Place the biosensor chip into isopropyl alcohol solution and sonicate the chip in isopropyl alcohol for 5 min in sonication bath. Remove the liquid from the surface of the chip by blotting the chip in paper Kim wipes.
4. Place the biosensor chip into distilled water and sonicate the chip for 5 min in sonication bath. Remove the liquid from the surface of the chip by blotting the chip in paper Kim wipes.
5. Repeat **step 3** and sonicate the biosensor chip in distilled water for 5 min in sonication bath. Remove the liquid from the surface of the chip by blotting the chip in paper Kim wipes.
6. Gently dry the cleaned biosensor chip with nitrogen.
7. Treat the dry biosensor chip with UV plasma for 15 min.
8. Prepare 2 μL anti-GFP (Abcam) solution diluted in 100 μL PBS.
9. Place a drop of anti-GFP solution onto a clean biosensor chip and incubate at 4 °C in a humidity-controlled environment overnight (~16 h) (*see* **Note 14**).
10. Rinse the biosensor chip with 10 mL of sterile PBS by dilution rinse in standard plastic 24-well plate (*see* **Note 15**).
11. Place 1 mL of PBS with 2% BSA to the biosensor chip in the well in standard plastic 24-well plate. Block the biosensor chip surface for 2 h at 4 °C in a humidity-controlled environment shaking.
12. Perform the dilution rinse as described in **step 3**.
13. Place 1 mL of 2–2.5 mg/mL protein extract from Subheading 3.3, **step 5** to the biosensor chip in the well in standard plastic 24-well plate, and incubate for 4 h at 4 °C in a humidity-controlled environment shaking.

14. Rinse the receptor-modified biosensor chips with 10 mL of sterile PBS by dilution rinse in standard plastic 24-well plate as described in **step 3**.
15. Gently dry the rinsed receptor-modified biosensor chip with nitrogen to remove the liquid, and place the receptor-modified biosensor chip onto a biosensor platform (*see* **Note 16**). At this point, the biosensor chip is immobilized with purified receptor kinase and can be used for investigating binding affinity and kinetics of the ligand-receptor pair [13, 16].

4 Notes

1. Using PVPP is preferable instead of polyvinylpyrrolidone (PVP) for phenolic compound removal because it can be easily removed from the sample at the filtration and centrifugation steps.
2. We recommend generating stable *Arabidopsis* plants expressing epitope-tagged full-length receptor kinase by using genomic DNA constructs driven by their own promoters. It is important to determine if an epitope-tagged receptor is functional by checking its ability to complement the respective mutant phenotype and by checking that it is expressed in the correct cellular location. Note that we also use the kinase-deleted version of an epitope-tagged receptor kinase, especially for the interaction studies with peptide ligands since the kinase domain of receptors is not required for ligand binding and removal of the kinase domain increases receptor stability [12, 17–19].
3. Densely sow seeds on one 150 mm plate to prepare approximately 1 g (fresh weight) of 12-day-old *Arabidopsis* seedlings required for one IP.
4. We have stored the powder sample for 3–4 months without any apparent protein degradation. To perform IP of several samples at the same time, it is practical to grind samples in advance and keep them in a −80 °C freezer.
5. This is generally more than enough, but if you have many transformations to do, use more.
6. **Step 3** can be repeated to remove traces of antibiotic, which will kill the leaf tissue after infiltration.
7. The rates of transformation are dependent on the viability of the plant and *Agrobacterium*. Therefore, it is important to repeat infiltrations in two midrib regions on 2–3 leaves in the same plant and use at least 6–8 independent healthy plants.
8. The expression of sufficient amounts of receptor kinase is one of the most important steps for the success of various biochemical

analysis including interaction studies of receptor kinases and peptide ligands. As such, we also use tobacco (*Nicotiana benthamiana*) plants that transiently express epitope-tagged receptors instead of *Arabidopsis* lines to isolate microsomal fractions for high-efficiency expression [11–13]. Proper subcellular localization and expression levels need to be monitored at a different time after infiltration to prevent the formation of aggregates which may cause false interactions [20]. It is also necessary to use good negative controls for following biochemical analysis. Two days after infiltration is generally a good time to harvest tobacco samples.

9. If the flow rate is slow, you can pipet up and down several times as necessary. In addition to the centrifugation step, this extra step is required to remove cell wall debris and PVPP efficiently for tobacco leaves.
10. The sample should be on ice while performing sonication to prevent overheating; keep probes away from the sample-air interface to minimize foaming.
11. Each IP should contain the same protein concentration in order to obtain consistent results, and depending on the affinity of the antibody, it may be necessary to adjust incubation times for optimal binding.
12. For the following biochemical analysis involving an interaction study with peptide ligands, we add peptides in the binding buffer (50 mM MES-KOH, pH 5.5 with 100 mM sucrose) to the purified receptor by IP and incubate with rotation for 0.5–1 h at 4 °C. Optimization of incubation time, binding, and washing conditions need to be examined extensively to prevent false protein-protein interactions using good positive and negative controls.
13. Do not touch the chip surface with paper to remove the liquid. Tap gently the edge of the biosensor chip with paper Kim wipes.
14. In drop incubation, make sure that the anti-GFP solution covers surface of the biosensor chip and that the incubation is done in humidity-controlled environment to prevent the liquid evaporation.
15. While doing dilution rinsing, always keep some liquid in wells and diminish surface drying to reduce the drying-induced antibody denaturation. Do not touch the chip surface to remove liquid.
16. Carefully remove all liquid from the gold electrode on the down-facing side of biosensor chip. Receptor coverage and alignment can be examined by using atomic force microscopy.

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