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Biochemical Purification of Native Immune Protein Complexes

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Abstract

Protein complex purification represents a powerful approach to identify novel players in plant innate immunity. However, the identification of interacting protein partners within a natural context has been a challenge for researchers. In this chapter, we describe a method of immunoaffinity chromatography using purified, antibodies to isolate native protein complexes from wild-type tissue. We detail the antibody purification and immobilization steps in addition to the co-immunoprecipitation protocol. In addition, a method to prepare protein samples for mass spectroscopy analysis is described. This straightforward protocol has been used to isolate and identify novel components of *Arabidopsis* immunity-associated protein complexes.

Keywords

Immunoprecipitation; Protein complex; Immune complex; Affinity chromatography

1. Introduction

Most biological responses require active adjustments of the cellular proteome. Emerging proteomics technologies have permitted the direct, large-scale analysis of proteins within the cell, which is a necessary component of attaining a systems understanding of plant biology. The earliest cellular responses to a given stimulus are frequently achieved through rapid alterations in protein activity and function via posttranslational regulatory modifications, changes in subcellular localization, and dynamic associations with interacting partners. Proteins are social entities; they interact with each other and work together to achieve necessary changes within each cell and the organism as a whole. However, the identification of interacting protein partners within a natural context has been a challenge for researchers. The elucidation of native protein complex composition *in vivo* and the dynamic changes that occur during active cellular signaling situations will greatly enhance our understanding of how plants respond to their environment.

In recent years, it has been demonstrated that many proteins involved in the plant innate immune response exist as members of multiprotein complexes within the cell. The molecular chaperone HSP90 (Heat Shock Protein 90 kD) (1) and co-chaperones SGT1 (Suppressor of G2 allele of *skp1*) (2) and RAR1 (Required for MLa12 Resistance) (3) are positive regulators of immune signaling. These chaperones interact with each other and various isoforms associate in different complexes with multiple disease resistance (R) proteins (1, 4–6). Moreover, specific R proteins have differential requirements of SGT1 and RAR1 for protein accumulation and activation of defense responses (6–9), indicating that distinct pools of these chaperone-R protein complexes exist within the plant cell. Additional components of immunity-associated protein complexes are now emerging. For example, a rice Rac/Rop GTPase, OsRac1, interacts with HSP90 and RAR1, but not SGT1, to regulate basal defense responses in rice (10). Most of the studies cited above utilized yeast two-

hybrid library screening to identify novel interactions, and/or in vitro or in vivo overexpression and co-immunoprecipitation to explore potential interactions (1, 2, 5, 10). The ability to identify novel protein interactions in vivo under native expression levels will enhance investigations into defense signaling pathways.

Mass spectrometry-based techniques for protein identification and characterization have emerged as a powerful tool to examine complex protein samples (11). This technology represents an excellent platform to identify novel protein–protein interactions through the affinity purification of target proteins and subsequent identification of associated proteins. Many purification tools and protocols have been developed using this general strategy. Small epitope tags such as the hemagglutinin (HA) or FLAG epitopes are commonly fused to protein coding sequences and transformed into *Arabidopsis* to investigate protein function, but these tags alone may not be suitable for protein complex purification due to moderate to high amounts of contaminants resulting from washing constraints. In an accompanying chapter, Qi and colleagues describe an affinity purification strategy using a high-affinity biotinylated tag that seeks to address this issue. Additionally, various tandem affinity purification (TAP) schemes have been developed for use in plants that employ a two-step affinity purification protocol in order to isolate protein complexes at a higher purity (12–14). However, many TAP systems use relatively large protein fusion tags and multiple purification steps that could disrupt native complex formation and integrity.

Alternatively, the isolation of protein complexes can be achieved by using antibodies raised against the target protein of interest. This strategy precludes the construction of a tagged transgene and functional validation of the tagged protein in transformed plants. Moreover, the affinity purification of protein complexes in a wild-type plant background assures that native protein complex formation has been preserved in the experimental plant material. The ability to directly isolate wild-type protein complexes under natural expression levels is arguably the most biologically relevant method for complex purification.

The *Arabidopsis* protein RIN4 has been identified as an important component of plant immune responses. RIN4 is localized to the plasma membrane and associates with at least two R proteins, RPS2 and RPM1, which monitor RIN4 for modifications induced by bacterial pathogen effector proteins (15–17). RIN4 associates with both RPS2 and RPM1 and negatively regulates these R proteins in the absence of their cognate effectors. In addition, RIN4 also negatively regulates basal defense responses and *rin4* mutant plants exhibit enhanced disease resistance in the absence of RPS2 and RPM1 (18). Due to these interesting aspects of RIN4 function at two levels of the plant innate immune response, a strategy was developed in order to identify additional components of the RIN4 immunity-associated protein complex.

In this chapter, we describe a protocol for isolating native protein complexes from *Arabidopsis thaliana*. The methods that are outlined were established from our experiments with the RIN4 protein and we have successfully used this protocol to isolate additional immune complexes (19). Essentially, polyclonal antibodies are affinity purified and immobilized on protein A or G beads. These antibody beads are used to isolate native-level protein complexes containing the target protein by immunoaffinity chromatography. The entire co-immunoprecipitated sample is subjected to mass spectrometry analysis in order to identify individual complex constituents. This straightforward method represents a powerful tool for native protein complex purification and characterization, and can easily be modified to purify other native protein complexes *in planta*.

2. Materials

2.1. Immobilization of Antigen for Affinity Purification of Antibodies

1. Purified antigen (2–3 mL of 5–10 mg/mL).
2. Cyanogen Bromide-activated sepharose 4B (GE Healthcare).
3. 1 mM HCl.
4. Coupling buffer: 100 mM NaHCO₃, 500 mM NaCl, pH to 8.3 with NaOH.
5. Blocking buffer: 100 mM Tris, 500 mM NaCl, pH 8.0.
6. Acetate buffer: 100 mM NaOAc, 500 mM NaCl, pH to 4.0 with Glacial Acetic acid.

2.2. Affinity Purification of Antibody

1. Immobilized antigen (Subheading 3.1).
2. Crude antiserum to the protein of interest.
3. 20 mL glass econo-column (BioRad).
4. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM NaH₂PO₄, pH 7.4.
5. Wash buffer A: 50 mM Tris, 120 mM HCl, 0.5% NP-40, pH 8.0.
6. Wash buffer B: 50 mM Tris, 1 M LiCl, 0.5% NP-40, pH 8.0.
7. Elution buffer: 50 mM Glycine-Cl, NaCl 150 mM, pH 2.5.
8. 2 M Tris.
9. 2% NaN₃ (sodium azide) in water.

2.3. Immobilization of Antibody on Protein A-Sepharose

1. Affinity-purified antibody (Subheading 2.3).
2. Protein A sepharose (GE Healthcare).
3. Cross-linking buffer: 200 mM sodium borate (Na₂B₂O₇), pH 9.0.
4. Dimethyl pimelimidate (DMP) solid.
5. Wash buffer: 200 mM ethanolamine, pH 8.0.
6. Final buffer: 0.01% Merthiolate in PBS.

2.4. *Arabidopsis thaliana* Growth Conditions

1. Four-week-old *Arabidopsis* plants, grown vegetatively in a controlled environmental chamber at 24°C with a 10 h-light/14 h-dark photoperiod under a light intensity of 85 µE/m²/s.

2.5. Protein Extraction and Immunoprecipitation

1. 20 mL glass econo-column (BioRad).
2. Immobilized affinity-purified antibody (Subheading 2.4).
3. 0.45 µm HPF Millex-HV high particulate filter, syringe-driven (Millipore).
4. 10 mL syringe.

5. Immunoprecipitation (IP) buffer 1 (extraction): 50 mM HEPES, 50 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, 1× Complete Protease inhibitor cocktail (Roche), 0.1 mg/mL Dextran (Sigma D1037), pH7.5 (see Note 1).
6. IP buffer 2 (low salt wash): 50 mM HEPES, 50 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, pH 7.5.
7. IP buffer 3 (high salt wash): 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, pH 7.5.
8. Phosphate buffer: 10 mM potassium phosphate, 50 mM NaCl, pH 6.8.
9. Elution buffer (low pH): 50 mM Glycine-Cl, 150 mM NaCl, 0.1% Triton X-100, pH 2.5.
10. Neutralization buffer: 2 M Tris.
11. Strataclean Resin (Agilent).
12. 3× Laemmli buffer: 188 mM Tris, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, 0.003% bromophenol blue, pH 6.8.

2.6. Sample Analysis

1. SDS-PAGE and Western Blotting equipment and reagents.
2. SilverQuest Silver Staining Kit (Invitrogen).

2.7. In-gel Trypsin Digest for Mass Spectrometry

1. Novex Colloidal Blue Stain Kit (Invitrogen).
2. Vacuum centrifuge.
3. Ultrasonic waterbath.
4. Wash buffer: 100 mM ammonium bicarbonate, NH_4HCO_3 .
5. 100% Acetonitrile.
6. Reducing buffer: 10 mM DTT in 100 mM NH_4HCO_3 .
7. Alkylating buffer: 55 mM iodoacetamide, 100 mM NH_4HCO_3 (iodoacetamide is highly toxic and care should be taken to reduce exposure).
8. Digestion buffer: 13 ng/ μL Sequencing grade modified trypsin (Promega) in 50 mM NH_4HCO_3 .
9. 60% Acetonitrile, 1% Trifluoroacetic acid in water.
10. 0.1% Trifluoroacetic acid in water.

3. Methods

Although mass spectrometry technology breakthroughs have greatly enhanced the amount of information that can be obtained from complex protein samples, the success of this powerful tool is dependent largely on the quality of protein samples delivered into the machine. Rapid and simple tissue processing can guarantee the reliability of results across multiple experimental replicates. The ability of newer mass spectrometers to analyze increasingly complex samples allows the researcher to isolate protein complexes with minimal

Inclusion of Dextran (MW = 400,000–500,000) in IP buffer 1 can reduce nonspecific protein–protein interactions.

purification steps, which favors the preservation of intact complexes. The analysis of all precipitated proteins directly via mass spectrometry without a SDS-PAGE band excision step can facilitate the identification of low abundance interactors. If high quality mass spectrometers are not available, individual SDS-PAGE band excision or additional liquid chromatography (e.g., MuDPIT) steps prior to loading on the machine may be necessary.

Experimental replication is a critical aspect of this method. We commonly use three biological replicates for each experiment. In order to be classified as a bona fide interacting protein during data analysis, the protein should be reliably identified in every experimental replicate and never in the negative control samples. With appropriate control samples, background spectra can easily be subtracted from the test samples. As a negative control, knockout lines in your protein of interest should be used to account for nonspecific binding. If a knockout line is unavailable, wild-type tissue extracts can be subjected to protein A or G sepharose precipitation to control for nonspecific binding. If applicable, the ability to troubleshoot the protocol with a known interacting protein will facilitate the success of the experiments. After each experimental replicate, the presence of your target protein should be verified in the elution fraction prior to mass spectrometry analysis.

The methods described below have been used to reproducibly isolate native-level protein complexes *in planta*. Before purification of protein complexes, polyclonal antibodies must be affinity purified in order to ensure the maximum capture of your target protein and to reduce nonspecific binding. The general format for affinity purification includes immobilizing the antigen on sepharose beads and using this to purify antibodies from crude antisera. Affinity purification of the polyclonal antibodies with purified target protein will result in higher complex purity during co-immunoprecipitation. Purified antibodies are cross-linked to Protein A-sepharose to facilitate column chromatography steps. Co-immunoprecipitation is performed with minimal tissue processing. Sample preparation for mass spectrometry analysis is also described. Steps critical for troubleshooting each method are also highlighted.

3.1. Antigen Immobilization for Affinity Purification of Antibody

This section describes the cross-linking of purified target protein to sepharose beads which will subsequently be used to affinity purify antibodies.

1. Weigh 1.0 g CN-Br-activated sepharose 4B and swell powder for 15 min at room temperature (RT) in 15-mL conical tube with 15 mL of 1 mM HCl (see Note 2).
2. Centrifuge at 1,000 *g* for 1–2 min at RT.
3. Wash swollen gel four times with 15 mL of 1 mM HCl. Spin as described in step 2 and remove supernatant between washes.
4. Wash the gel with 2–3 mL coupling buffer, centrifuge at 1,000 *g* and remove supernatant. *Immediately* add protein (2–3 mL of 5–10 mg/mL) to be coupled. Add coupling buffer to the tube to a final volume of 10 mL (see Note 3).
5. Incubate protein and gel solution end-over-end at 4°C overnight. Remove a 20 µL aliquot, boil in 1× Laemmli buffer, and use supernatant to check coupling efficiency by running an SDS-PAGE gel (see Note 4).

Insoluble proteins can be coupled in coupling buffer containing 2 M urea.

Either recombinant protein or peptide antigen can be used. Recombinant proteins should be at least 80% pure. All steps with coupling buffer should be completed without delay because reactive groups hydrolyze at the coupling pH.

Coupling efficiency can be verified by boiling a small aliquot of beads in 1× Laemmli buffer and running on an SDS-PAGE gel. When compared to the input, little or no protein should be observed after coupling is completed.

6. Centrifuge sample at 1,000 *g* for 1–2 min at RT. Re-suspend in 10 mL of coupling buffer and add 1/10 bed volume of blocking buffer. Block remaining active groups for 2 h at RT.
7. Wash excess non-covalently bound protein with 5 mL coupling buffer followed by 5 mL acetate buffer. Wash alternately in coupling and acetate buffers three to four times, ending with coupling buffer. The sample should be centrifuged at 1,000 *g*, 1–2 min at RT between wash steps.
8. Store protein-sepharose conjugate at 4°C in 5 mL PBS with 0.02% Na-azide until ready for use (see Note 5).

3.2. Affinity Purification of Antibody

This step describes the purification of polyclonal antibodies from crude antisera that will be subsequently immobilized and used for co-immunoprecipitation of the target protein.

1. Incubate 3 mL protein-sepharose conjugate “beads” from Subheading 3.1 with 40 mL crude antiserum in a 50 mL tube rotating end-over-end overnight at 4°C (see Note 6).
2. All steps below should be performed with ice-cold buffers.
3. Load serum/beads mixture into BioRad econo-column, collect flow through and save for analysis (see Note 7). Rinse tube twice with 10 mL PBS and transfer to column.
4. Wash column with 20 mL Wash buffer A.
5. Wash column with 20 mL Wash buffer B.
6. Wash column with 20 mL Wash buffer A.
7. Wash column with 20 mL PBS.
8. Add 7.5 μ L of 2 M Tris buffer to 25 1.5 mL tubes (to neutralize eluted fractions).
9. Elute column twice by adding 5 mL of elution buffer each time and collecting 500 μ L fractions. Mix tubes to neutralize pH.
10. Quantify the antibody in each tube via Bradford assay. Generate two pools of purified antibody: one low concentration (0.25 mg/mL) and one high concentration (>0.5 mg/mL).
11. Add 2% Na-azide to 0.02% final concentration. Antibody can be aliquoted and stored at 4°C or –20°C for long-term storage.
12. Wash column twice with 10 mL PBS. Cap the column and add 1 mL PBS with 0.02% Na-azide to protein-beads. Column can be stored at 4°C (see Note 5).

3.3. Antibody Immobilization on Protein A-Sepharose

This step describes the generation of antibody beads used during the immunoaffinity chromatography to isolate protein complexes.

1. Incubate 2 mg affinity-purified antibody with 1 mL Protein A-sepharose in 10 mL PBS buffer end-over-end for 1 h at RT (see Note 8).

The protein-sepharose conjugate can be stored for years at 4°C and can be repeatedly used for antibody purifications. The amount of serum used depends on the titer of the antibody. For highly antigenic proteins (>10,000 titer based on ELISA results for polyclonal antibodies) 15 mL of antisera should be sufficient. The flow through sera can be saved and used for western blotting experiments.

2. Centrifuge $3000 \times g$ for 5 min and wash beads twice with 10 mL cross-linking buffer.
3. Re-suspend beads in 10 mL cross-linking buffer and remove 100 μ L of bead suspension for analysis (step 9).
4. To initiate cross-linking reaction, add DMP (solid) to final concentration of 20 mM. (see Note 9).
5. Incubate 30 min end-over-end at RT. Remove 100 μ L for analysis (step 9).
6. Centrifuge at $3,000 \times g$ for 5 min and discard supernatant. To stop the reaction, wash beads once with 10 mL wash buffer, centrifuge at $3,000 \times g$ for 5 min and discard supernatant.
7. Add 10 mL fresh wash buffer and incubate for 2 h end-over-end at RT.
8. Centrifuge and re-suspend in 10 mL Final buffer. Antibody beads can be stored at 4°C (see Note 10).
9. Check cross-linking efficiency by boiling samples before (step 3) and after (step 5) cross-linking in 1× Laemmli buffer. (see Note 11).

3.4. Plant Growth and Tissue Collection

1. *Arabidopsis* plants are grown vegetatively in a controlled environment chamber (see Note 12).
2. Collect 5 g of *Arabidopsis* leaf tissue in foil, freeze in liquid N₂, and store at –80°C (see Note 13).

3.5. Protein Extraction and Immunoprecipitation

The quality and reproducibility of mass spectrometry data relies heavily on the quality of the protein samples that are delivered for analysis. Therefore, much care should be taken during buffer preparation and tissue processing. We have found it most useful to include the simplest number of steps during tissue processing and co-immunoprecipitation in order to ensure the most reliable and consistent results across experimental replicates.

1. All steps should be conducted in a cold room at 4°C with ice-cold buffers (see Note 14). All centrifugation steps should be conducted at 4°C.
2. Grind 5 g of leaf tissue in liquid N₂ with mortar and pestle. Re-suspend in 11 mL IP Buffer 1.
3. Centrifuge at $10,000 \times g$ for 10 min at 4°C.

Depending on type of antibody IgG, either protein A- or protein G-sepharose should be used. Refer to (20). Protein A-sepharose should be used for all antibodies produced in rabbit.

Cross-linking must be performed at pH > 8.3 to be efficient.

Antibody beads can be stored at 4°C for at least 1 year and used multiple times.

Successful cross-linking should result in absence of heavy chain bands (55 kD) in bead supernatant after boiling.

We use 4-week-old *Arabidopsis* plants for most of our experiments. Exact growth conditions and tissue treatments depend on your target protein and should be determined empirically.

If the target protein is localized to the chloroplast or mitochondria, purification of intact organelles prior to complex purification will reduce the number of contaminating proteins.

For protein extraction and immunoprecipitation, use freshly sterilized buffers made specifically for MS analysis that are free of common contaminants such as keratin. Buffers must be absolutely free of contamination in order to obtain robust mass spectrometry data.

4. During centrifugation of cell lysate, wash antibody beads in IP buffer 1 (without protease inhibitors). Centrifuge antibody beads at 3,000 rpm for 3 min, discard supernatant, re-suspend in IP buffer 1 to wash. Repeat (see Note 15).
5. Filter cell lysate supernatant using a 0.45 μm high particulate filter and syringe. Remove a 50 μL aliquot of input sample and save for analysis (see Note 16).
6. Add 500 μL antibody-coupled beads to filtered lysate. Incubate end-over-end for 2.5 h at 4°C (see Note 17).
7. Load material onto glass column. Pass flowthrough through column twice. Remove a 50 μL aliquot of the final flowthrough and save for analysis (see Note 16).
8. Wash column twice with 20 mL IP buffer 2 (low salt wash).
9. Wash column twice with 10 mL IP buffer 3 (high salt wash). Remove a 50 μL aliquot of the high salt wash flowthrough and save for analysis (see Note 16).
10. Wash column with 5 mL Phosphate buffer.
11. Elute bound proteins four times with 1 mL Elution buffer. Let buffer sit on column for 2 min before each elution. Collect each fraction and neutralize with 15 μL 2 M Tris.
12. Combine elution fractions and concentrate with Strataclean Resin. Add 20 μL of Strataclean resin to the pooled elution and incubate the sample end-over-end for 20 min at RT. Centrifuge at $5,000 \times g$ for 2 min, remove supernatant, and re-suspend resin in 40 μL of 1 $\times$ Laemmli buffer (see Note 18).
13. Elute protein from Strataclean resin by boiling in 40 μL 1 $\times$ Laemmli buffer. Centrifuge sample at $10,000 \times g$ for 1 min, transfer supernatant to a new 1.5 mL tube and store at -20°C .
14. To recharge antibody beads in the column, wash column with 30 mL (3×10 mL washes) Elution buffer. Neutralize column with 30 mL (3×10 mL) phosphate buffer. Store in PBS at 4°C (see Note 19).

3.6. Sample Assessment

1. Verify immunoprecipitation of target protein and any known interactors by running 5 μL of the sample on a 12% SDS-PAGE gel followed by western blotting.
2. Visualize co-immunoprecipitated proteins by running 5 μL of the sample on a 12% SDS-PAGE gel and stain using SilverQuest Silver Staining Kit (Invitrogen) (Fig. 1).

We include Dextran protein in IP buffer 1 during complex binding but not during wash and elution steps. The addition of Dextran acts to decrease nonspecific protein binding. If nonspecific interactions are a problem, incubate antibody beads with IP Buffer 1 containing Dextran for 15 min to pre-block beads.

Save aliquots of the cell lysate input, flow through, and high salt wash for troubleshooting purposes. Boil aliquots in 1 $\times$ Laemmli and subject to SDS-PAGE and western blotting to visualize target protein and any known interactors at each step of the process. This information can be used to optimize immunoprecipitation conditions for the target protein complex. Depending on the target protein, it may be necessary to troubleshoot ideal IP buffer conditions. Solubility of target proteins can be achieved by increasing detergent concentrations as well as including different detergents. Nonspecific protein binding can be decreased by increasing the salt concentration. We have never purified interacting proteins when the salt concentration was increased above 300 mM.

It is ideal to use the shortest incubation time possible during this step to reduce nonspecific binding.

It is possible to precipitate proteins with a traditional Trichloroacetic Acid protocol. We have found that using Strataclean resin is more reproducible and results in higher yields than traditional precipitation protocols.

The low pH (Elution buffer) column washes must be completed rapidly to avoid denaturing the antibodies. The column can be reused multiple times (we use up to six times). We separate the columns and use one column for a specific genotype.

3.7. Sample Preparation for Mass Spectrometry

1. Run the remaining 30 μL sample on a 10% SDS-PAGE gel. Run sample about 6 mM into the separating gel.
2. Stain by colloidal coomassie (Invitrogen kit).
3. Remove protein sample smear with razor and perform an in-gel trypsin digest (Subheading 3.8) (see Note 20).

3.8. In-Gel Trypsin Digestion

1. Chop gel pieces into 1 mM fragments and transfer to a 1.5-mL tube.
2. Wash gel pieces with 100 μL Wash buffer for 5 min (see Note 21).
3. Discard buffer and add 50 μL 100% acetonitrile and dehydrate at RT for 15 min (see Note 22).
4. Remove acetonitrile and dry completely in a vacuum centrifuge for approximately 15 min (gel pieces should turn completely white).
5. Rehydrate the gel pieces with 50 μL Reducing buffer. Heat at 56°C for 30 min to reduce the sample.
6. Remove reducing buffer and add 50 μL 100% acetonitrile. Incubate for 3–5 min at RT. Repeat twice.
7. Dry in vacuum centrifuge for 15 min.
8. Add 50 μL Alkylating buffer to alkylate cysteine residues. Incubate for 20 min in dark at RT.
9. Discard supernatant and wash briefly with 50 μL Wash buffer.
10. Remove buffer and add 50 μL Wash buffer and incubate for 15 min at RT (see Note 23).
11. Remove liquid and add 50 μL 100% acetonitrile. Incubate for 15 min at RT.
12. Dry completely in vacuum centrifuge.
13. Rehydrate gel pieces in 30–50 μL Digestion buffer. Add enough digestion buffer to cover the gel pieces. Add more Digestion buffer if gel pieces absorb all the liquid.
14. Allow gel pieces to absorb digestion buffer (see Note 24).
15. Incubate at 37 C overnight.
16. Spin sample down, collect liquid into new 1.5 mL tube, and save.
17. Add 15–30 μL of 60% Acetonitrile, 1% trifluoroacetic acid in water to each gel piece.
18. Sonicate gel pieces in ultrasonic waterbath for 10 min.
19. Centrifuge tubes at $21,000 \times g$ for 30 s.

Alternatively, the sample can be sent to a mass spectrometry facility for sample preparation. All solutions should be made fresh each time. Ensure that solutions are sterilized and free of common contaminants such as keratin. The gel piece will turn white upon dehydration. Repeat if gel piece is not completely dehydrated. If the gel pieces are not completely destained, wash with 1:1 acetonitrile: Wash buffer solution at 37°C for 30 min. Incubate samples at 37°C for 15–25 min to facilitate absorption of the digestion buffer. If all the digestion buffer is absorbed by the gel pieces after the 15–25 min at 37°C, then add 10–30 μL of 0.5 $\times$ Wash buffer (enough to cover samples).

20. Collect supernatant and add to solution from step 17.
21. Vacuum centrifuge solution from step 26 until *almost* dry (see Note 25).
22. Add 5–20 μ L of 0.1% Trifluoroacetic acid to the tubes and sonicate tube in Branson 1200 waterbath for 5 min.
23. Centrifuge tubes at $21,000 \times g$ for 30 s.
24. Store samples at 4 C if it will be analyzed within a few days or freeze at -80 C (see Note 26).

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Do not dry out the samples completely or for an extended amount of time.
Freezing the samples can result in sample loss.

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Fig. 1.

Isolating the RIN4 protein complex. Affinity-purified RIN4 antiserum coupled to protein A was used to purify the RIN4 protein complex from 5 g of Arabidopsis leaf tissue. **(a)** Silver stained gel of elutions. The double mutant *rps2-101c/rin4* was used as a negative control, while the transgenic line expressing *nproRPS2:HA* in the *rps2-101c* background was used as a positive control. *Asterisks* and *circles* indicate bands corresponding to the molecular weight of RIN4 and RPS2:HA, respectively. **(b)** Western blots of eluted fractions.