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Title: Removal and Replacement of Endogenous Ligands from Lipid-Bound Proteins and Allergens

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements outside of the filming date. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **37**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Geoffrey A.Mueller**: Many allergens bind hydrophobic molecules. This protocol enables the complete removal and replacement of these ligands, allowing us to study their impact on structure and immunogenicity in a systematic manner **[1]**.

1.1.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Peter M.Thompson**: The use of reverse-phase HPLC coupled with thermal annealing has two advantages - it removes endogenous ligands and it helps to solublize ligands to open up otherwise inaccessible binding sites **[1]**.

1.2.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Geoffrey A.Mueller**: If we can determine the factors that contribute to allergenicity, we may be able to better design therapies that avoid these factors **[1]**.

1.3.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.4. **Alexander C.Y. Foo**: Many proteins bind lipid ligands, which are strongly retained and may influence both structure and function. Our method allows the systematic study of these interactions **[1]**.

1.4.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.5. **Peter M. Thomspon**: The limited solubility and accessibility of many lipid cargoes may inhibit the loading process. Be sure to take care when preparing these ligands and to consider the need for system-specific adaptations **[1]**.

1.5.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.6. **Alexander C.Y. Foo**: As working with hydrophobic and insoluble ligands is an adjustment for some allergists and biochemists, it can be useful to see what the samples look like at different stages **[1]**.

1.6.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Endogenous Lipid Removal

2.1. After cloning, expression, and purification, apply 12 milliliters of the cleaved Bla g 1 (blah G one) to a centrifugal filter unit **[1-TXT** with a less than 10-kilodalton molecular weight cut-off for multiple centrifugations in a swing-bucket rotor until the total volume has been reduced to less than 2 milliliters **[2-TXT]**.

2.1.1. WIDE: Talent adding product to unit **TEXT: See text for Bla g 1 cloning and elution details** Author NOTE: step 2.1.1 is repeated in 3.4.2 and 4.1.1. We shot this scene once for the sake of convenience.

2.1.2. Talent adding unit to centrifuge **TEXT: 10-15 min, 5000 x g, RT**

2.2. Load the resulting concentrate onto a 250- x 10-millimeter HPLC (H-P-L-C) system equipped with a C18 reverse-phase chromatography column equilibrated with 97% buffer A and 3% buffer B **[1-TXT]**.

2.2.1. Talent loading concentrate onto system **TEXT: HPLC: high pressure liquid chromatography**

2.3. Elute Bla g 1 at a 1.5-4 milliliter/minute flow rate as indicated in the Table **[1]**, using the fluorescence absorbance at 280 nanometers to monitor the elution process **[2-TXT]**.

2.3.1. LAB MEDIA: Table 1

2.3.2. Talent monitoring elution process/Talent setting abs to 280 nm **TEXT: Refer to Figure 1B in text manuscript**
Author NOTE: We opted for a still image for step 2.3.2 instead of a video. The manipulations described could not be recorded due to our inability to screen capture the computer interface. It may be useful to insert a reference to Figure 1 B, which depicts the elution process in question and includes all relevant notation

2.4. Starting at around 34-40 minutes and a buffer B concentration above 74%, collect no more than 4 milliliters of each Bla g 1 fraction **[1-TXT]**.

2.4.1. Fraction being collected **TEXT: See text for all buffer preparation details**

2.5. Aliquot the pooled Bla g 1 fraction into glass test tubes, cover the tubes with paraffin film, and perforate the film with two holes **[1]**.

2.5.1. Talent adding fraction to tube, covering tube, perforating film *Videographer: Can split action into separate shots as necessary*

2.6. Then freeze the samples at minus 80 degrees Celsius for 1 hour **[1-TXT]** and use a lyophilizer to dry the resulting delipidated protein samples **[2-TXT]**.

2.6.1. Talent placing tube(s) into -80 °C storage **TEXT: Alternative: Freeze in LN_2 with tube rotation to avoid tube breakage**

2.6.2. Talent lyophilizing sample **TEXT: Optional: Stored dried protein for several months at 4 °C**

3. Apo-Reconstitution and Bla g 1 Cargo-Loading

3.1. To determine the anticipated Bla g 1 yield, resuspend a lyophilized, delipidated test aliquot in 5 milliliters of refolding buffer **[1]**.

3.1.1. WIDE: Talent resuspended sample in buffer, with buffer container visible in frame *Videographer: Important step*

3.2. Heat the mixture in 500-milliliter beaker containing 250 milliliters of water to 95 degrees Celsius on a hot plate with stirring **[1]** and intermittent vortexing **[2]**.

3.2.1. Talent placing solution into water bath on hot plate *Videographer: Important step*

3.2.2. Talent vortexing sample *Videographer: Important step*

3.3. Hold the solution at 95 degree for 30-60 minutes **[1]** before allowing the water bath to slowly equilibrate to room temperature **[2-TXT]**.

3.3.1. Mixture being stirred *Videographer: Important step*

3.3.2. Talent turning off hot plate *Videographer: Important step* **TEXT: Optional: Store annealed protein over night at 4 °C**

3.4. When the solution has cooled, pass the annealed Bla g 1-lipid mixture through a 0.22-micron syringe filter to remove particulate matter **[1]** and use a new centrifugal filter with a 10-kilodalton cutoff to buffer exchange the filtered protein three times into PBS to remove any residual free fatty acids and organic solvent **[2]**.

3.4.1. Talent passing mixture through syringe filter *Videographer: Important step*

3.4.2. Talent adding mixture to centrifugal filter, with PBS container visible in frame *Videographer: Important step* Author NOTE: step 2.1.1 is repeated in 3.4.2 and 4.1.1. We shot this scene once for the sake of convenience.

3.5. Then assess the protein concentration using a standard protein analysis assay to determine the anticipated yield for the remaining Bla g 1 aliquots **[1]**.

3.5.1. Talent opening BCA kit, with sample visible in frame

3.6. To reconstitute the Apo-Bla g 1, resuspend the Bla g 1 aliquots in 5 milliliters of refolding buffer per aliquot **[1]** and anneal the sample as demonstrated **[2]**.

Author NOTE: 3.6 describes the same annealing process as 3.1-3.3. As such, we did not film this stage. Media from 3.1-3.3 can be re-used if needed (3.6.1 is equivalent to 3.1.1, 3.6.2 is equivalent to 3.2.1)

3.6.1. Talent adding buffer to sample, with buffer container visible in frame
3.6.2. Talent placing tube into beaker on hot plate

3.7. To load the Bla g 1 with phospholipids, add 10 milligram/milliliter stock of the desired cargo to chloroform inside a glass test tube **[1]** and evaporate the chloroform to produce a lipid film **[2]**.

3.7.1. Talent adding cargo to chloroform, with cargo chloroform containers visible in frame
3.7.2. Talent evaporating chloroform **TEXT: Fatty acid ligand loading: Use 20 mM stock in methanol or DMSO**

3.8. Then add PBS to the tube to produce a final phospholipid concentration of 20-millimolar **[1]**.

3.8.1. Talent adding PBS to tube, with PBS container visible in frame NOTE: 3.8.1 – 3.9.1 in one shot

3.9. Heating the phospholipid above the phase transition temperature of the lipid cargo to rehydrate the lipid film **[1]** and vortex until the solution turns cloudy **[2-TXT]**.

3.9.1. Talent heating phospholipid *Videographer: Difficult step*
3.9.2. Sample being vortexed *Videographer: Difficult step* **TEXT: 100 W, 42 kHz sonication may be required to fully resuspend/rehydrate from cargos**

3.10. Then add the phospholipid cargo to produce a 20x molar excess of ligands relative to Bla g 1 based on the anticipated yield **[1-TXT]**. A precipitate my form **[2]**.

3.10.1. Shot of cargo being added solution to solution *Videographer: Important/difficult step* **TEXT: Total organic solvent volume should not exceed 2%**
3.10.2. Shot of precipitate *Videographer: Important/difficult step*

3.11. Vortex to mix **[1]** before annealing the protein as demonstrated **[2]**.

3.11.1. Talent vortexing
3.11.2. Sample being removed from beaker on hot plate to show final product

4. Phospholipid Cargo Removal/Loading Confirmation

4.1. To confirm cargo removal or loading by phosphorus-31 NMR (N-M-R), use a centrifugal filter to concentrate the sample to greater than 100 micromolar as demonstrated **[1-TXT]**.

4.1.1. WIDE: Talent adding sample to filter **TEXT: NMR: nuclear magnetic resonance** Author NOTE: step 2.1.1 is repeated in 3.4.2 and 4.1.1. We shot this scene once for the sake of convenience.

4.2. Prepare reference phospholipid samples of known concentrations in PBS buffer as indicated **[1-TXT]** and dilute the Bla g 1 and reference to a 1:1 ratio with cholate buffer to a total volume of about 600 microliters **[2]**.

4.2.1. Talent adding sample to PBS, with PBS container visible in frame **TEXT: Final concentrations: 2, 1.5., 1. 0.5, and 0.25 mM**
4.2.2. Talent adding buffer to sample, with buffer container visible in frame

4.3. Use a broadband probe to acquire one-dimensional 31-phosphorus-NMR spectra of the cholate-solubilized Bla g 1 samples and reference phospholipid standards **[1]** and compare the Bla g 1 31-phosphorus-NMR spectra **[2]** to those obtained for the phospholipid reference samples to confirm the removal of endogenously bound ligands **[3]** and/or the binding of the desired ligands based on the chemical shifts of the visible peaks **[4]**.

4.3.1. Talent adding probe to NMR instrument
4.3.2. LAB MEDIA: Figure 2A
4.3.3. LAB MEDIA: Figure 2A *Video Editor: please emphasize black data line*
4.3.4. LAB MEDIA: Figure 2A *Video Editor: please emphasize red data line*

4.4. Then compare the peak intensity of the Bla g 1 spectrum to that of the phospholipid reference standards **[1]** to allow confirmation of the full binding stoichiometry **[2]**.

4.4.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize data curves in top graph*
4.4.2. LAB MEDIA: Figure 2C *Video Editor: please emphasize data line in bottom graph*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
3.1.-3.4., 3.10.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.9., 3.10. The limited solubility of most hydrophobic ligands represents a major barrier to this procedure. As such, care must be taken to ensure that the ligands are uniformly dispersed throughout the refolding process, maximize accessibility and facilitating binding to the Bla g 1 cavity. In the case of phospholipids, additional steps (as illustrated in 3.7-3.10) are required to minimize the formation of large insoluble aggregates. The careful heating and cooling described in the annealing step further enhances ligand solubility while increasing access to the otherwise buried Bla g 1 binding cavity.

Results

5. Results: Representative Bla g 1 Purification, Lipid Removal Verification, and Recovery

5.1. Using affinity chromatography, recombinant GST (G-S-T)-Bla g 1 can be readily isolated to a high level of purity **[1-TXT]**, producing a yield of 2-4 milligrams/liter of cell culture **[2]**.

5.1.1. LAB MEDIA: Figure 1A *Video Editor: please emphasize GST-Bla g 1 text and arrow and/or band indicated by GST-Bla g 1 text and arrow* **TEXT: GST: glutathione S-transferase**

5.1.2. LAB MEDIA: Figure 1A

5.2. Overnight incubation with TEV (T-E-V) protease at 4 degrees Celsius is sufficient to remove the GST tag, yielding the final product at approximately 24 kilodaltons **[1-TXT]**.

5.2.1. LAB MEDIA: Figure 1A *Video Editor: please emphasize Bla g 1 text and arrow and/or band indicated by GST-Bla g 1 text and arrow* **TEXT: TEV: tobacco etch virus**

5.3. Applying the Bla g 1 to a reverse-phase C18 column yields a distinctive elution profile **[1]**, with two large peaks at 50% buffer B **[2]**, and a second large peak at 75% buffer B **[3]**.

5.3.1. LAB MEDIA: Figure 1B

5.3.2. LAB MEDIA: Figure 1B *Video Editor: please emphasize H1 and H2 peaks*

5.3.3. LAB MEDIA: Figure 1B *Video Editor: please emphasize H4 peak*

5.4. Phosphorus-31 NMR spectra of Apo-Bla g 1 show no detectable phospholipids **[1]**.

5.4.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize black data line*

5.5. A standard curve can be produced from the NMR using reference samples of known DSPC (D-S-P-C) concentrations **[1-TXT]**.

5.5.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize sequentially add/emphasize data lines from bottom to top of graph top graph* **TEXT: DSPC: distearoylphosphatidylcholine**

5.6. Comparing the phosphorus-31 signal intensity obtained from DSPC-Bla g 1 against this standard curve can be used to yield the binding stoichiometry of the lipids per protein **[1]**.

5.6.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize data line in bottom graph*

5.7. Circular dichroism spectra for Apo- and lipid-loaded Bla g 1 show minima of 220 and 210 nanometers **[1]**, indicative of a predominantly alpha-helical structure **[2]**.

5.7.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize inverse peaks at 210 and 220*

5.7.2. LAB MEDIA: Figure 3A

5.8. Circular dichroism-based thermal denaturation assays also show a cooperative loss of alpha-helical secondary structure, indicative of a folded globular domain **[1]**.

5.8.1. LAB MEDIA: Figure 3B *Video Editor: please emphasize red data line*

5.9. In addition, analysis of the resulting melting temperatures reveals a significant increase upon nMix ligand binding consistent with Bla g 1 obtained from its natural allergen source **[1-TXT]**.

5.9.1. LAB MEDIA: Figure 3C *Video Editor: please emphasize orange data bar* **TEXT: nMix: natural Bla g 1 ligands (*i.e.*, palmitate, stearate, and oleate)**

Conclusion

6. Conclusion Interview Statements

6.1. **Geoffrey A. Mueller**: The success of this protocol relies on its ability to overcome both the limited solubility of hydrophobic ligands and the inaccessible nature of the Bla g 1 binding cavity **[1]**.

6.1.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.3., 3.9.)

6.2. **Alexander C.Y.Foo**: This procedure lays the groundwork for immunological studies, such as T cell proliferation assays, to assess the effect of lipid ligands on sensitization, and the molecular mechanisms through which this occurs **[1]**.

6.2.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.3. **Peter M. Thompson**: Coupling this procedure with further biophysical assays, we have shown that the binding of hydrophobic ligands enhances Bla g 1 stability, with potential downstream implications for epitope generation and allergenicity **[1]**.

6.3.1. LAB MEDIA: To be provided by Authors: Named talent says the statement above in an interview-style shot, looking slightly off-camera