

Analysis of Calcium-Induced Conformational Changes in Calcium-Binding Allergens and Quantitative Determination of Their IgE Binding Properties

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Abstract

The polcalcin family is one of the most epidemiologically relevant families of calcium-binding allergens. Polcalcins are potent plant allergens that contain one or several EF-hand motifs and their allergenicity is primarily associated with the Ca^{2+} -bound form of the protein. Conformation, stability, as well as IgE recognition of calcium-binding allergens greatly depend on the presence of protein-bound calcium ions.

We describe a protocol that uses three techniques (SDS-PAGE, circular dichroism spectroscopy, and ELISA) to describe the effects that calcium has on the structural changes in an allergen and its IgE binding properties.

Key words: Allergen, Polcalcin, Calcium, EF-hand, IgE binding

1. Introduction

Calcium-binding allergens include a wide variety of proteins that have been isolated from pollen, parasites, and fish (1). These allergens are highly cross-reactive and include proteins that have a wide variety of structural, functional, and calcium-binding properties.

One of the most epidemiologically relevant families of calcium-binding allergens is the polcalcin family, which includes pollen allergens from grasses, trees, and weeds. All these allergens bind calcium through interactions with EF-hand motifs. The polcalcin family contains at least 35 pollen allergens having two, three, or

four EF-hand motifs (1). These motifs consist of two perpendicularly placed α -helices and one interhelical loop forming a single calcium-binding site (2). Calcium binding to EF-hand motifs has shown to alter the secondary and tertiary structure of the proteins because there is a change in the orientation of the two amphipathic α -helices flanking the binding motif (3). These conformational changes might affect the allergen's stability as well as its IgE binding properties (4–7). A detailed knowledge of these molecular changes may be highly significant for the molecular design of hypoallergenic variants that can be used in allergen-specific immunotherapy.

This chapter contains a detailed protocol of three methods used to analyze the effect of calcium on the structural and IgE binding properties of a given allergen. Here, we focused on the analysis of the Cup a 4 protein, an allergen from the Arizona Cypress that was recently described in our laboratory (8). The polcalcin family Cup a 4 protein contains 4 EF-hand motifs and is highly cross-reactive with other calcium-binding allergens (unpublished results). The calcium binding to the Cup a 4 protein was proven by its different migration rate on SDS-PAGE in the presence and absence of calcium. A more detailed secondary structure analysis was carried out through circular dichroism spectroscopy. Finally, the IgE binding capability of the allergen in the presence or absence of calcium is quantitatively measured by an ELISA assay.

2. Materials

All solutions are prepared using deionized water and analytical grade reagents. Procedures are carried out at room temperature unless indicated otherwise. Basic chemicals were purchased from Sigma Aldrich unless indicated otherwise.

2.1. Dialysis

1. Normal dialysis buffer: 25 mM phosphate buffer pH 7.4.
2. High calcium dialysis buffer: 25 mM phosphate buffer pH 7.4 + 250 μ M CaCl_2 .
3. Calcium-free dialysis buffer: 25 mM phosphate buffer pH 7.4 + 250 μ M EGTA (see Note 1).
4. Regenerated cellulose dialysis membranes with the appropriate molecular weight cut-off (Spectrum Europe B.V., Breda, The Netherlands).
5. Standard dialysis membrane closures (Spectrum Europe B.V.).
6. Dialysis reservoir, magnetic stirrer.

2.2. SDS-PAGE Electrophoresis

1. Stacking gel buffer: Tris-HCl 0.5 M, pH 6.8. Weigh 6.1 g Tris, dissolve in 75 ml water, and adjust pH with HCl. Add water up to 100 ml. Store at room temperature.
2. Resolving gel Buffer: Tris-HCl 1.5 M, pH 8.8. Weigh 18.1 g Tris, dissolve in 75 ml water, and adjust pH with HCl. Add water up to 100 ml. Store at room temperature.
3. Acrylamide/bisacrylamide solution (30%) was purchased from Biorad Laboratories.
4. Ammonium persulfate was used as a 10% solution in water. Prepare a fresh 1 ml solution and store at 4°C for up to 1 month (see Note 2).
5. *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED).
6. 10% SDS solution in deionized water (see Note 3).
7. 5× Electrophoresis running buffer: 0.125 M Tris-HCl, 0.96 M glycine, 0.5% SDS. Weigh 15.12 g Tris, 72.06 g glycine and dissolve in 800 ml water. Add 5 ml SDS 10% and complete to 1 L with water. Store at room temperature.
8. 2× sample loading buffer: 0.125 M Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.04% bromophenol blue, 20% glycerol. In a 15 ml Falcon tube add 2.5 ml Tris-HCl 0.5 M pH 6.8, 4 ml SDS 10%, 0.3 ml EDTA 0.5 M pH 8, 1 ml β-mercaptoethanol (98%), 2.29 ml glycerol (87%), and 0.4 mg Bromophenol blue. Complete with water up to 10 ml. Freeze at -20°C in 1 ml aliquots (see Note 4).
9. SDS-PAGE was performed using the Mini-PROTEAN Tetra Electrophoresis System (Biorad).
10. Broad range molecular weight markers (Biorad).
11. Staining solution: 45% methanol, 7.5% acetic acid, 0.25% Coomassie brilliant blue (CBB) R250. Dissolve 2.5 g CBB in 450 ml methanol, add 75 ml glacial acetic acid, and complete with water up to 1 L.
12. Destaining solution I: 45% methanol, 7.5% acetic acid. In a graduated cylinder, add 450 ml methanol, 75 ml glacial acetic acid and complete with water up to 1 L.
13. Destaining solution II: 5% methanol, 7.5% acetic acid. In a graduated cylinder, add 50 ml methanol, 75 ml glacial acetic acid and complete with water up to 1 L.

2.3. Circular Dichroism Spectroscopy

1. Far-UV capable spectropolarimeter fitted with a thermostated cell holder and interfaced with a computer (see Note 5).
2. Spectrosil precision 0.1 cm cells.

2.4. ELISA Assay

1. 96 well flat bottom Nunc MaxiSorp™ plates (Nalge Nunc International, Rochester, NY, USA).

2. Phosphate-Buffered Saline (PBS) pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 2 mM KH_2PO_4 .
3. Coating solutions: PBS+250 μM calcium (add 1.25 ml of 100 mM CaCl_2 to 50 ml 10 $\times$ PBS, complete with water to 500 ml), PBS+250 μM EGTA (add 250 μl EGTA 0.5 M pH8 to 50 ml 10 $\times$ PBS, complete with water to 500 ml).
4. Washing solutions: PBS+0.5% Tween, PBS+250 μM calcium+0.5% Tween, PBS+250 μM EGTA+0.5% Tween.
5. Blocking solutions: PBS+0.5% Tween+5% Bovine Serum Albumin (BSA), PBS+250 μM calcium+0.5% Tween+5% BSA, PBS+250 μM EGTA+0.5% Tween+5% BSA.
6. Antibody diluting solutions: PBS+1% Tween+10% BSA, PBS+500 μM calcium+1% Tween+10% BSA, PBS+500 μM EGTA+1% Tween+10% BSA (see Note 6).
7. HRP conjugated anti-IgE antibody (Southern Biotech, AB, USA).
8. Substrate solution: Add four Dako OPD tablet (Dako Denmark A/S, Copenhagen) to 12 ml water, allow to dissolve in the dark, then add 12 μl H_2O_2 per added tablet (see Note 7).
9. 1 M H_2SO_4 solution: Prepare by adding 53.3 ml of 95–97% H_2SO_4 to 946.7 ml prechilled deionized water (see Note 8).

3. Methods

3.1. Sample Preparation

For all assays a purified allergen is needed. The allergen can originate from natural or recombinant sources and can be purified using several techniques (described in refs. 8–10). The protein of interest will be analyzed under three conditions: normal calcium, high calcium, and calcium-free. We have defined normal calcium as the status in which solutions are prepared for day-to-day use in the lab. High calcium conditions imply the presence of 250 μM CaCl_2 and in calcium-free buffers, EGTA is added to a final concentration of 250 μM .

1. Soak an appropriate length of dialysis tubing in deionized water for 20–30 min (see Note 9).
2. Clamp one end of the dialysis tube with a standard closure. Fill the tubing with water and check for leaks (see Note 10). Load the sample into the tube and leave space for a small bubble and clamp the other end. Check for leaks.
3. Immerse the clamped tube that contains the sample in a dialysis reservoir containing 2 L of the appropriate dialysis buffer (normal calcium, high calcium, or no calcium), check that the

clamped tube has slightly positive buoyancy, and add a magnetic stir bar. Place on a magnetic stirrer and set to low speed (see Note 11) in a cold room at 4°C.

4. Change dialysis buffer after 2 and 5 h and then leave overnight. Perform one final buffer change the next day and dialyze for an additional 2 h.
5. Carefully open one of the clamps, quantitate, and aliquot the dialyzed sample (see Note 12).

3.2. SDS-PAGE

1. Clean glass plates with a mild detergent and rinse with ethanol. Dry with a laboratory wipe. Set up glass plates in gel casting chamber.
2. 12% Resolving gel preparation: In a 15 ml polystyrene tube, mix 2.5 ml resolving buffer, 3.3 ml water, and 4 ml 30% acrylamide/bisacrylamide solution. Add 100 µl 10% SDS and mix gently by inversion. Add 50 µl APS 10% solution and 5 µl TEMED, and immediately add 4 ml to gel casting chamber. Overlay with 1 ml water. When gels have polymerized and immediately before stacking gel preparation, invert the casting chamber to pour out the overlaying water and gently dry with Whatman® paper.
3. Stacking gel preparation: Mix 1.24 ml stacking buffer with 3 ml water and 0.65 ml 30% acrylamide/bisacrylamide solution. Add 50 µl SDS 10% and mix gently by inversion. Add 25 µl APS 10% and 5 µl TEMED and pour immediately into gel casting chamber containing polymerized resolving gel. Insert a 10-well comb.
4. Sample preparation: To 1–2 µg dialyzed protein, add an equal volume of 2× sample loading buffer. Heat samples at 95°C for 5 min, transfer immediately to ice, and briefly spin in a microfuge to collect condensate.
5. Load samples in gel and add molecular weight marker. Run at 50 V for 5 min and then raise voltage to 200 V. The electrophoresis is finished when the dye front reaches the bottom of the gel.
6. Open the gel plates with the use of a plastic spatula and carefully transfer the gel into an appropriately sized container. Add staining solution, cover the container with aluminum foil, and stain for 20–30 min in an orbital shaker.
7. Discard staining solution, add destaining solution I, and incubate in an orbital shaker for 2–3 h, replacing the destaining solution after the first hour. Replace destaining solution I with destaining solution II and keep overnight at 4°C.
8. Analyze results: If the protein of interest binds calcium, different migration patterns may be observed (Fig. 1).

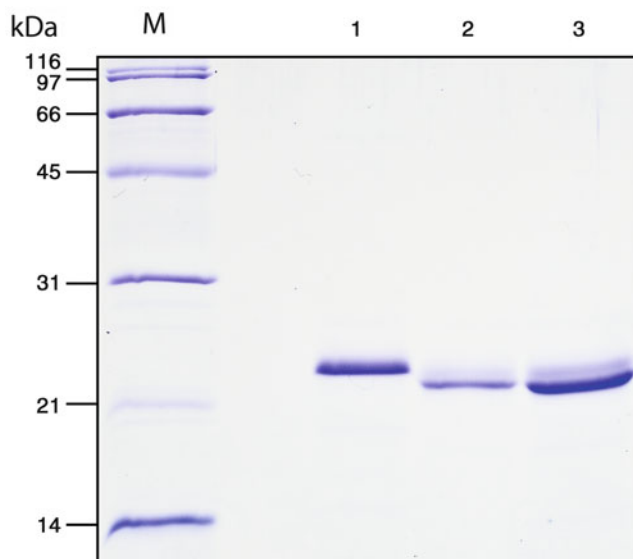


Fig. 1. SDS-PAGE analysis of Cup a 4 in the presence/absence of calcium. Lane 1. 250 μ M EGTA. Lane 2. Normal calcium. Lane 3. 250 μ M CaCl_2 . 1 μ g protein was loaded in each well. M: Broad range molecular weight markers (Biorad).

3.3. Circular Dichroism Spectroscopy

1. Prepare 25 μ M dilutions of each of the protein samples: Normal, calcium-free (250 μ M EGTA), and high calcium (250 μ M CaCl_2) (see Note 13).
2. Switch on the CD spectrometer (see Note 5) and allow time for the electronics and lamp to stabilize (approximately 30 min).
3. Load the cell with protein sample and collect a CD spectrum using the following parameters: 25°C, 190–250 nm range at a rate of 50 nm/min, a bandwidth of 1.0 nm, and a response time of 2 s.
4. Collect three spectra per sample and calculate the mean spectrum.
5. Repeat steps 3 and 4 with the buffer used for each protein sample.
6. Subtract the buffer spectrum from the protein sample spectrum.
7. Normally, the spectrum ellipticity, θ_λ , is obtained in millidegrees; therefore it is necessary to convert the results to molecular ellipticity, $[\theta]_\lambda$, using the following formula:

$$[\theta]_\lambda = \frac{100 \cdot \theta_\lambda}{C \cdot l}$$

where C is the molar concentration in mol/l and l is the cell length in cm.

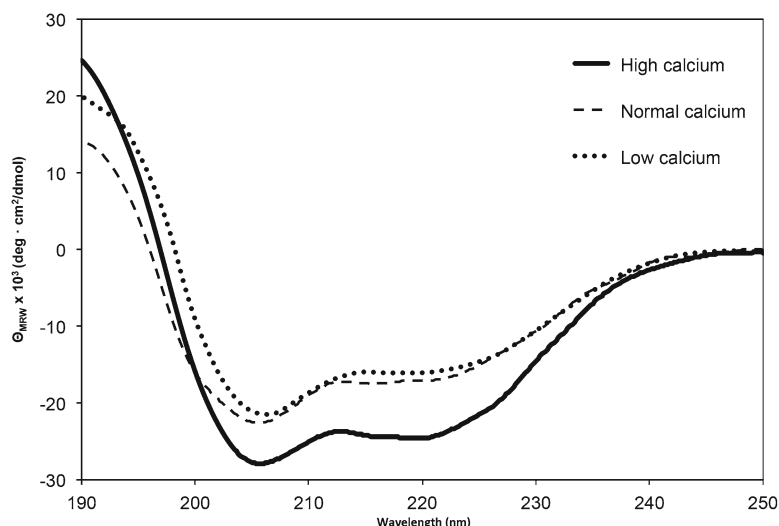


Fig. 2. Far UV CD spectra of 20 μM Cup a 4 in the presence/absence of calcium.

8. Plot-normal, no-calcium, and high-calcium spectra (Fig. 2 and Note 14).

3.4. Quantitative Analysis of IgE Binding Properties

All steps are carried out at room temperature unless stated otherwise.

1. Antigen coating: The allergen of interest is prepared at a final concentration of 7 $\mu\text{g}/\text{ml}$ in one of the following solutions: PBS, PBS+250 μM calcium, or PBS+250 μM EGTA. Add 100 μl per well to a 96-well microplate (see Note 15). The number of wells depends on the number of sera to be analyzed, taking into account that each analysis will be performed in duplicate. Cover and incubate overnight at 4°C on a flat surface.
2. Empty each plate by inversion and dry by blotting on a paper towel. Wash three times by adding 200 μl of the selected washing solution and incubating for 5 min on a plate shaker at 200 rpm (see Note 16). Use the washing solution that corresponds to the calcium content selected for each assay (normal, no calcium, or high calcium).
3. Block for 2 h in 200 μl of the corresponding blocking solution (normal, no calcium, or high calcium). Remove blocking solutions by inversion and dry by blotting on a paper towel.
4. Prepare serum dilutions: Dilute each serum 1:1 in each of the antibody diluting solutions (normal, no calcium, or high calcium). Add 100 μl of each diluted serum and incubate for 2 h on a plate shaker at 200 rpm.
5. Empty each plate by inversion and dry by blotting on a paper towel. Wash three times as explained in step 2.

6. Add 100 μ l of secondary antibody solution. This solution is prepared by diluting the anti-IgE antibody 1:800 in the corresponding blocking solution (normal, no calcium, or high calcium). Incubate for 1 h on a plate shaker at 200 rpm.
7. Empty each plate by inversion and dry by blotting on a paper towel. Wash three times as explained in step 2.
8. Add 100 μ l substrate solution to each well and incubate for 20–30 min in the dark (see Note 17).
9. Stop reaction by adding 50 μ l of 1 M H_2SO_4 per well (see Note 18).
10. Read 450 nm absorbance in a 96-well micro plate reader.
11. Analyze results: Calculate the mean and standard deviation of absorbance values obtained for the five negative control serum samples in each of the calcium conditions. The final absorbance value for each sample is calculated by the following formula:

$$\text{Abs}_n = \text{Abs} - (\text{Abs}_{\text{neg}} + 2\text{SD}),$$

where Abs_n is the normalized absorbance value, Abs is the O.D. value for each sample, Abs_{neg} is the mean absorbance calculated for the five negative control sera, and SD is the standard deviation calculated for the five negative control sera. The Abs_n calculated by this method are considered reactive against the tested allergen when they are positive. Plot the Abs_n values (Fig. 3).

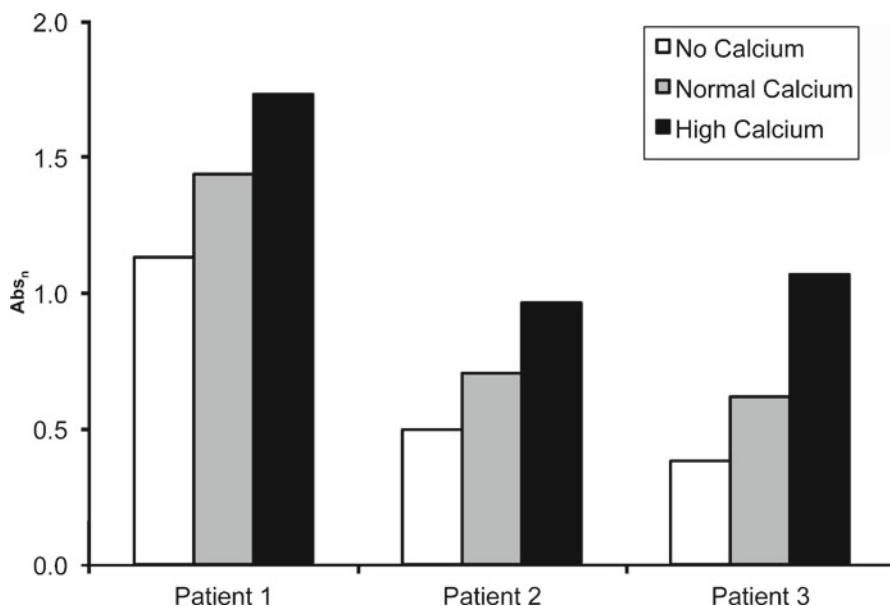


Fig. 3. IgE binding activity of sera from three allergic patients to Cup a 4 in the three calcium conditions: high, normal, and no calcium. Patients were selected in the context of the EU CRAFT Cyprall project QLK-CT-2002-71661, serum was obtained after written consent. All patients were positive by skin prick test to *C. arizonica* extract prepared in native conditions.

4. Notes

1. The best way to prepare these buffers is using 0.1 M phosphate buffer pH 7.4 (11): Add 250 ml to three graduated cylinders. Complete the first one to 1 L with water to obtain normal dialysis buffer. Add 2.5 ml 100 mM CaCl_2 to the second one and complete to 1 L with water (high-calcium dialysis buffer). Add 500 μl EGTA 0.5 M, pH8 and complete to 1 L with water to obtain calcium-free dialysis buffer.
2. Prepare 10 ml of a 10% solution by adding 1 g ammonium persulfate to 10 ml water, and store aliquots at -20°C . Once thawed, APS solution can be stored at $+4^\circ\text{C}$ for 1 month.
3. An easy way to prepare 10% SDS without frothing is by weighing 50 g of SDS and adding it on top of a beaker containing 350 ml water. The next day, the SDS will be dissolved and can be completed to 500 ml with water. Always wear a mask while weighing SDS.
4. This buffer can be prepared at $4\times$ when the protein concentration of the sample is low. This allows loading of larger sample volumes.
5. We use a JASCO J-600 spectropolarimeter (Jasco Europe SLR, Cremella, Italy) and a NESLAB RTE-100 water bath (ThermoElectron Measurement Systems, Karlsruhe, Germany).
6. Since the sera will be diluted 1:1, the detergent and blocking agent concentrations in these solutions are doubled.
7. 12 ml is enough for one 96-well plate, and scale up or down, accordingly. This solution must be used within 60 min and should be kept in the dark. The original Dako instructions suggest adding a much lower quantity of H_2O_2 (5 μl). We find, however, that it is better to use 12 μl in order to avoid the reduction of activity that takes place after long-term storage of H_2O_2 . This solution is toxic to aquatic organisms, and should be disposed according to local regulations.
8. Wear gloves and protect your eyes with safety goggles while preparing this solution. Dilution of concentrated acid should always be done in a fume hood. To avoid strong exothermic reactions, add concentrated acid to water slowly; never add water to a concentrated acid.
9. We use the Dialysis Tubing Calculator at the Spectrum Laboratories Web site: <http://www.spectrumlabs.com/dialysis/dtCalc.html>. Always use gloves to handle the dialysis membrane because the membrane is susceptible to cellulolytic microorganisms.

10. Always perform all dialysis sample handling procedures over a beaker in case the membrane slips. Use a paper towel to check for leaks: gently set the clamp with the tube on a paper towel and observe if moisture appears.
11. It is very important to check the dialysis frequently during the first hour to make sure that the membrane is not moving too quickly or touching the magnetic stirrer in the bottom of the dialysis reservoir.
12. If the protein is going to be used only for ELISA or SDS-PAGE analysis, quantitation by colorimetric methods (i.e., Bradford, BCA, or Lowry) is precise enough. However, if CD analysis is going to be performed, a more precise quantitation method based on A280 with an empirically determined extinction coefficient (12) is recommended.
13. Take into account that the protein samples were dialyzed against buffers that contained 500 μM EGTA and CaCl_2 . Dilute with the appropriate buffer in each case. We recommend a first dilution step with 25 mM pH 7.4 phosphate buffer to correctly adjust calcium and EGTA concentrations. Samples can then be diluted to the final concentration with the appropriate buffer.
14. Further analysis of the secondary structure data can be performed using the dichroweb server (13) and analyzed as has been previously described (14).
15. The number of wells used depends on the number of sera to be analyzed. For each serum, three conditions will be tested (250 μM EGTA, residual calcium, and 250 μM calcium) and duplicates should be performed; count at least six wells per sample. For practical reasons each calcium condition is performed on a separate plate, include at least five nonreactive negative control sera as well as two no serum control wells in each plate.
16. We have found that washing in a $23 \times 16 \times 8$ cm plastic food container (wash bowl) yields the lowest background and highest reproducibility results: Fill a wash bowl with 200 ml washing buffer and place the ELISA plate inside. Push it down so that all the wells are covered by the washing buffer, move the wash bowl sideways three or four times, then invert the plate in a sink, and blot dry with a paper towel. Perform this step three times and use a different wash bowl for each calcium condition.
17. The developing reaction can be carried out on the bench top, while the plates are covered with Styrofoam boxes. Check every 5 min until sufficient color is observed.
18. When working with a large number of samples, it is recommended to pipet the sulfuric acid in the same order as the developing solution was pipetted.

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