

One-step Affinity Purification of the Chloroplast ClpP Complex from the Green Alga *Chlamydomonas reinhardtii* Using the Strep-tagII Epitope Tag

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[Abstract] This protocol describes the affinity purification of the chloroplast ClpP complex from *Chlamydomonas reinhardtii*. To this purpose, we have created a *Chlamydomonas reinhardtii* strain in which the chloroplast encoded ClpP1 subunit of the ClpP complex is tagged at its C-terminal end by a strep-tag II peptide.

Materials and Reagents

1. Strain: The strain ClpP1-Strep has been described in (Derrien *et al.*, 2012) and can be obtained from the ChlamyStation culture collection (<http://chlamystation.free.fr>)
2. StrepTrap HP 1 ml (GE Healthcare, catalog number: 28-9075-46)
3. EDTA free protease inhibitors (Roche, catalog number: 05 056 489 001)
4. d-Desthiobiotin ≥98% (IBA, catalog number: 2-1000-005)
5. 1 M Tris HCl pH 8.0 at 4 °C
6. Ammonium sulphate saturated solution (>4.5 M; undissolved salt will settle to the bottom)
7. 0.4 M EDTA (pH 8.0)
8. 5 M NaCl
9. DTT
10. 1 M MgCl₂
11. Glycerol
12. Buffer A (see Recipes)
13. Buffer B (see Recipes)
14. Elution buffer (see Recipes)

Equipment

1. Beckman centrifuge, rotors (JA-10, JA-20, JA-12 and Type 70ti) and polycarbonate centrifuge tubes (Beckman Coulter)
2. Semi-continuous centrifuge (e.g. Firlabo)

3. FPLC apparatus (BioLogic DuoFlow, Bio-Rad Laboratories)
4. French Press (Aminco)
5. Rotary wheel

Procedure

A. *Chlamydomonas reinhardtii* cell culture

1. Grow 5 L of a *Chlamydomonas reinhardtii* culture (strain) in TAP medium (Recipe available at the *Chlamydomonas* Resource Center <http://chlamycollection.org/media-recipes/>) under constant illumination (40 $\mu\text{E}/\text{m}^2/\text{s}$) at 25 °C, in a 5 L stirred Erlenmeyer flasks with air bubbling. Harvest cells when culture density corresponds to late exponential phase (cell density comprised between 6×10^6 and 10^7 cells/ml). 5 L cultures are inoculated from pre-cultures in exponential grows phase (2 to 3×10^6 cells/ml). Cell density is measured by cell counting using a Malassez counting chamber. Knowing that *Chlamydomonas* generation time is ~8 h in the indicated conditions, you are able to determine the volume of the inoculum to obtain the desired concentration at the desired date and time.

B. Harvest cells

From here on, work at 4 °C with all solutions and glassware pre-cooled to 4 °C.

1. Concentrate cell culture by continuous centrifugation. In our Firlabo apparatus, the final volume is 1.5 L. Distribute this volume into three 500 ml centrifuge bottles.
2. Centrifuge at 5,000 rpm for 5 min at 4 °C in a Beckman JA-10 rotor.
3. Resuspend cells in a final volume of 500 ml of buffer A.
4. Centrifuge at 5,000 rpm for 5 min at 4 °C in a Beckman JA-10 rotor.
5. Resuspend cells using 10 ml of buffer A. At this stage, the solution is dense with a dark green color. Add proteases inhibitors according to the recommendations provided by Roche, and EDTA at a final concentration of 1 mM. Adjust final volume to 40 ml.

C. Prepare soluble cellular protein extract

1. Cell lysis: Fill up the pressure cell of the French Press with the 40 ml of cell suspension. To remove air from the pressure cell, tilt it in order to allow bubbles to reach the exit tap, open tap and push the piston until all the air has exited. Then close the tap and put the French Press under pressure (6,000 psi). Start cell lysis by slowly opening the tap of the pressure cell. Collect outflow in a 50 ml Falcon tube held on ice. During the process, liquid should flow slowly, to avoid sudden pressure drops.
2. Repeat this procedure once on the cell lysate.

3. Pre-clearing: transfer cell lysate into a centrifuge tube (Beckman Thickwall Polycarbonate, 50 ml, 29 x 104 mm) and centrifuge at 20,000 rpm for 30 min at 4 °C in a Beckman JA-20 rotor. Collect supernatant.
4. Ultracentrifugation: adjust to a final volume of 50 ml with buffer A. Add MgCl₂ to a final concentration of 6 mM (a minimal MgCl₂ concentration of 5 mM is required to allow stacking of thylakoid membranes, needed for their complete removal). Distribute the 50 ml in two ultracentrifuge tubes (Beckman capped tube, Polycarbonate, 26.3 ml, 25 x 89 mm). Centrifuge at 60,000 rpm for 60 min at 4 °C in a Beckman rotor Type 70ti.
5. Carefully collect supernatant (orange-yellow color) with a syringe equipped with a needle long enough to reach the bottom of the tube. Then transfer it in a 100 ml measuring cylinder.

D. Fractionate soluble protein fraction by ammonium sulphate precipitation

Note: This part of the protocol is specific to Chlamydomonas reinhardtii ClpP complex, if you want to adapt the protocol to another species, you have to determine the ammonium sulphate concentration range in which your protein complex of interest precipitates.

1. Measure the supernatant volume and add 1/3 volume of saturated ammonium sulphate solution to reach 25% saturation final. To avoid uneven precipitation, it is important to proceed progressively and under constant and gentle steering (place magnetic stir bar at bottom of cylinder). Then let the solution equilibrate for 15 min at 4 °C and transfer to a 50 ml Falcon tube.
2. Centrifuge at 5,000 rpm for 10 min at 4 °C in a Beckman JA-12 rotor.
3. Carefully pour off supernatant in a measuring cylinder (100 ml). Measure its volume and add 1/4 volume of saturated ammonium sulphate solution to reach 45% saturation final, taking the same precautions as above. Let the solution equilibrate for 15 min at 4 °C and transfer it into two 50 ml Falcon tube.
4. Centrifuge at 5,000 rpm for 10 min at 4 °C in a Beckman JA-12 rotor. Carefully pour off supernatant (pellet is soft). Then using a syringe and a needle, remove all remaining traces of supernatant.
5. Add 4 ml of buffer B onto each pellet and close caps. Put tubes on a rotary wheel at 4 °C and let the pellets solubilize (20-30 min at 20-30 rpm). Pool the two protein solutions into a 15 ml tube, if necessary adjust volume to 10 ml with buffer B and gently mix by inverting the tube.

E. Affinity chromatography on StrepTrap column.

This step can be performed using a FPLC apparatus, a peristaltic pump or even manually with a syringe.

1. Equilibrate the column in buffer B (10 column volume at 1 ml/min).
2. During this time, centrifuge the protein extract at 5,000 rpm for 5 min at 4 °C in a Beckman JA-20 rotor to remove potential insoluble particles that might clog the column.
3. Binding: load the protein extract on the column (0.5 ml/min; if column is operated manually, this corresponds to a flow of 1 drop per second).
4. Wash the column with buffer B (10 column volume at 1 ml/min).
5. Elute with buffer E (10 column volume at 1ml/min, fractionation: 1ml). Collect in fractions of 1 ml. Fraction 2 corresponds to the peak of ClpP concentration, but fractions 2 to 5 can be pooled to yield high purity ClpP complex (for quality control, check Figure 1 in Derrien *et al.* (2012), showing samples at each step of the purification process).

F. Concentration and storage.

1. Once concentrated, purified ClpP complex can be stored at -80°C for months. First, equilibrate a Centricon centrifugal filter unit (100 kDa cut-off) with 1 ml of buffer B. Then, pool elution fractions 2 to 5 and reduce volume to about 500 µl, add 500 µl of buffer B and homogenise the solution by pipeting gently (avoid bubble formation). If removal of Desthiobiotine is needed repeat this step several times in order to dilute the compound. Finally, concentrate solution to a volume of 200 µl to 300 µl. Concentration should be around 500 µg/ml (for quality control, check Figure 3 in Derrien *et al.* (2012), showing a Coomassie stained and a silver stained SDS-PAGE of the purified ClpP complex).

Recipes

1. Buffer A
 - 20 mM Tris-HCl pH 8.0 at 4 °C
 - 150 mM NaCl
 - 1 mM DTT
2. Buffer B
 - 20 mM Tris-HCl pH 8.0 at 4 °C
 - 150 mM NaCl
 - 10% glycerol
 - 1 mM DTT
3. Elution buffer
 - 20 mM Tris-HCl pH 8.0 at 4 °C
 - 150 mM NaCl

10% glycerol

2.5 mM Desthiobiotine

As desthiobiotine is poorly soluble in water, it is first solubilised in DMSO at 500 mM and then added to buffer B at the above-indicated concentration.

Note: It is important to adjust pH of the Tris HCl 1 M stock solution at 4 °C. The pH of Tris buffers increases as temperature decreases (a 20 degree fall corresponds to an increase of 0.5 to 0.6 pH units).

Acknowledgments

This protocol is adapted from and previously used in Derrien *et al.* (2012).

References

1. Derrien, B., Majeran, W., Effantin, G., Ebenezer, J., Friso, G., van Wijk, K. J., Steven, A. C., Maurizi, M. R. and Vallon, O. (2012). [The purification of the *Chlamydomonas reinhardtii* chloroplast ClpP complex: additional subunits and structural features.](#) *Plant Mol Biol* 80(2): 189-202.