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Rubisco Extraction and Purification from Diatoms

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[Abstract] This protocol describes a method to extract ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) from diatoms (*Bacillariophyta*) to determine catalytic performance. This protocol has been adapted from use in cyanobacteria and higher plants (Andrews, 1988; Whitney and Sharwood, 2007). First part (steps A1-A3) of the extraction provides a crude extract of Rubisco that is sufficient for carboxylation assays to measure the Michaelis constant for CO_2 (K_C) and the catalytic turnover rate (k_{cat}^c). However, the further purification steps outlined (steps B1-B4) are needed for measurements of Rubisco CO_2/O_2 Specificity ($S_{C/O}$, [Kane *et al.*, 1994]).

Keywords: Rubisco, Diatoms, Extraction, Phytoplankton, Carbon fixation

[Background] Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco, EC 4.1.1.39) catalyzes the first step in the photosynthetic assimilation of CO_2 and thus plays a fundamental role in photosynthesis and the global carbon cycle. Rubisco has been isolated from a wide range of organisms, from archaea, bacteria, algae to plants, and displays a diverse range of kinetics between organisms (Galmes *et al.*, 2014; Tcherkez *et al.*, 2006; Whitney *et al.*, 2011). Knowledge of Rubisco kinetics is a key component for understanding how photosynthesis and thus the biological sink of carbon will respond to rising anthropogenic CO_2. Diatoms are a group of unicellular algae responsible for ~20% of global photosynthesis (Falkowski and Raven, 2007) but as yet have been relatively poorly studied in terms of their Rubisco kinetics.

Isolation and purification of Rubisco is required before kinetic assays can be undertaken. Due to differences in cell structure and organic composition between organisms, the method for the purification of viable Rubisco enzyme needs to be continually optimized. This protocol describes a method to extract and purify Rubisco using size exclusion chromatography from diatoms in preparation for kinetic assays. The method is similar to Whitney and Sharwood (2007), used for the purification of Rubisco overexpressed in *E. coli,* in that a French press is used to mechanically rupture cells. The French press is necessary to obtain sufficient cell lysis as diatoms are unicellular with silica frustules, unlike plant tissue in which sufficient lysis is easily achieved by homogenizing frozen leaf tissue in a mortar and pestle. Furthermore, due to the low *in vivo* concentrations of Rubisco in diatoms (Losh *et al.*, 2013),

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large diatom culture volumes concentrated via centrifugation, are needed to obtain enough biomass compared to plant tissue and *E. coli* lines with overexpressed Rubisco.

Materials and Reagents

1. 15 ml centrifuge tubes with conical bottoms
2. 50 ml centrifuge tubes with conical bottoms
3. 500 ml centrifuge tubes with conical bottoms
4. 1.5 ml microcentrifuge tubes
5. 1 ml syringe
6. 1 ml Bio-Scale mini Macro-Prep high Q ion exchange column (Bio-Rad Laboratories, catalog number: 7324120)
7. Amicon Ultra-4 centrifugal filter (30,000 NMWL) (EMD Millipore, catalog number: UFC803024)
8. Amicon Ultra-100 centrifugal filter (100,000 NWML) (EMD Millipore, catalog number: UFC910024)
9. Diatoms
10. Liquid nitrogen
11. Polyvinylpolypyrrolidone (PVPP; insoluble) (Sigma-Aldrich, catalog number: 77627)
12. Additional materials To test for Rubisco activity (Optional):
    Labelled CO_2 (as $NaH^{14}CO_3$) (5 mCi) (PerkinElmer, catalog number: NEC086H005MC)
    Ribulose-1,5-bisphosphate (RuBP; synthesized, purified and stored anaerobically as described in Kane *et al.*, 1998)
13. Acetic acid (Sigma-Aldrich, catalog number: A9967 or 27225)
    Note: The product acetic acid (A9967) has been discontinued.
14. Methanol (Sigma-Aldrich, catalog number: M1770 or 494437)
    Note: The product Methanol (M1770) has been discontinued.
15. Soluble protein (as determined by Bradford assay) (Coomassie Plus Assay Kit) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 23236)
16. Additional materials to test for protein using Native and SDS-PAGE (Optional):
    Gel code blue stain (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 24590)
    4-12% Tris-glycine mini gels (Thermo Fisher Scientific, Invitrogen™, catalog number: XV04120PK20)
    4-12% Bis-Tris gels (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0321PK2)
    SDS reducing buffer for SDS-PAGE (see Recipes)
    TBS buffer for SDS-PAGE (see Recipes)
    AttoPhos reagent (Astral Scientific, Gymea, NSW, Australia)
    Antisera raised against the large subunit holoenzyme of Rubisco in *Phaeodactylum tricornutum*
    Alkaline Phosphatase conjugated secondary antibody

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17. 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) (Sigma-Aldrich, catalog number: E9502)
18. Ethylenediaminetetraacetic acid disodium salt (EDTA) (Sigma-Aldrich, catalog number: E5134)
19. Dithiothreitol (Sigma-Aldrich, catalog number: D0632)
20. Plant protease inhibitor cocktail (Sigma-Aldrich, catalog number: P9599)
21. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
22. Triethanolamine (Sigma-Aldrich, catalog number: 90279)
23. Magnesium acetate (Sigma-Aldrich, catalog number: M5661)
24. Glycerol (Sigma-Aldrich, catalog number: G5516)
25. Extraction buffer (see Recipes)
26. Column buffer (see Recipes)
27. Column elution buffer (see Recipes)
28. Specificity ($S_{C/O}$) buffer (see Recipes)

Note: All chemicals are of A.C.S. grade.

Equipment

1. French pressure cell press (Thermo Fisher Scientific, model: FA-078)
2. Coulter Counter Z Series (Beckman Coulter, model: Z Series Coulter Counter)
3. Centrifuge (large volumes [1 L] at 2,000 *x g*, small volumes [< 15 ml] at 17, 600 *x g*, 4 °C)
4. Fume hood
5. Superdex 200 (GE Healthcare, catalog number: 17517501)
6. FPLC (Äkta Pure 25) setup at 4 °C for size-exclusion chromatography using Superdex 200/30 (GE Healthcare, model: Äkta Pure 25)
7. To confirm purification and activity of Rubisco:
    a. Protein transfer apparatus and immunoblot imagining equipment – to check abundance and purity of extracted Rubisco
    b. Radioisotope laboratory and associated septum capped vials and syringes

Procedure

A. Total soluble cellular (crude) protein extraction – suitable for measurement of the Michaelis constant for CO_2 (K_C), catalytic turnover rate (k_{cat}^c) and the first step for CO_2/O_2 specificity assays ($S_{C/O}$).
    1. Diatoms are grown in 2 L batch cultures at 20 °C under continuous light (c. 150 µmol photons m^{-2} sec^{-1}) in sterile seawater to which 0.2 µM filtered nutrients, vitamins and trace metals were added to give the final concentrations as defined in the Aquil medium. For a full recipe and guidelines for making Aquil recipe, see Sunda *et al.*, 2005. Growth rates are monitored by cell counting using a Coulter Counter. During exponential growth, cells are concentrated to a pellet via gentle centrifugation (2,000 *x g* for 10 min) at 15 °C and the supernatant discarded.

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Approximately 1 g of a cell pellet is required to purify Rubisco for measuring $S_{C/O}$. To limit storage space, collect cells into 1.5 ml microcentrifuge tubes and then snap freeze in liquid nitrogen and store at -80 °C until further analysis.

2. The cell pellets are re-suspended in a total of 5 ml ice cold extraction buffer in a 15 ml polypropylene tube. The cells are ruptured using a pre-chilled French press at 140 MPa (see Figure 1 for photo and Video 1 of French press) and the cell lysate collected into the same tube.

Figure 1. Cell lysis using a French press. Photo of a French Pressure Cell that can efficiently lyse microalgae cells, including diatoms. Algae cell extracts in ice cold buffer are placed in the ice-cold stainless steel French Pressure Cell and hydraulic pressure applied. Once at 140 MPa the outlet to the Cell is slowly opened. The cells lyse as they emerge from high to ambient pressure and the cell extract is collected into a 15 ml or 50 ml polypropylene tubes. A slow flow rate of sample out of the Cell (~1 to 2 drops sec^{-1}) is maintained to ensure the pressure is maintained (see Video 1, acknowledgement Bratati Mukherjee).

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Video 1. Use of French press to lyse cells

3. To the cell lysate, add polyvinylpolypyrrolidone (1%, w/v) to bind secondary metabolites, which are then removed by centrifugation (17,600 *x g*, 4 °C, 5 min).
4. After centrifugation Rubisco remains fully intact as confirmed by native PAGE (see Data analysis, Figure 2) and fully soluble, remaining in the cell lysis supernatant as confirmed by SDS-PAGE (see Data analysis, Figure 3).
5. Rubisco k_{cat}^c (and K_C) is quantified by $^{14}CO_2$ fixation assays (see Data analysis).
   a. Rubisco in the soluble protein extract is activated for 10 to 15 min with 15 mM $NaH^{14}CO_3$ and 15 mM $MgCl_2$.
   b. In 7 ml septum capped glass scintillation vials 20 µl of the extract is assayed for 60 sec in 0.5 ml of 50 mM EPPS-NaOH pH 8.2, 10 mM $MgCl_2$, 0.6 mM RuBP, 10 µg ml^{-1} carbonic anhydrase and varying $NaH^{14}CO_3$ concentrations (1.2 to 12 mM which equates to ~15 to 150 µM $^{14}CO_2$ at pH 8.0 at 25 °C) (Sharwood *et al.*, 2016). The buffer and vials are equilibrated with the appropriate O_2/N_2 gas mixture prior to adding the $NaH^{14}CO_3$ and sample.
   c. The reaction is stopped with 0.2 ml 0.5 N formic acid and non-fixed $^{14}CO_2$ is vented by heating the vials at 85 °C in the fume hood.
   d. The dried residue is dissolved in 0.5 ml double distilled H_2O and vortexed with 1 ml Scintillant (Packard, Ultima Gold XR) before the labelled 3-phosphoglycerate (^{14}C) is measured in a scintillation counter. This crude extraction is suitable for measurements of Rubisco maximum carboxylation rate (V_{max}) and Rubisco Michaelis constant for CO_2 under N_2 (K_C) and 21:79% O_2:N_2 (K_c^{air}) (Sharwood *et al.*, 2008; Sharwood *et al.*, 2016). To quantify k_{cat}^c the V_{max} value is divided by the Rubisco content in the assay. Measuring Rubisco content is achieved using the ^{14}C-CABP binding method or by immunoblot analysis. The accuracy and experimental limitations of both approaches are detailed in Whitney and Sharwood, 2014. Purification of Rubisco is necessary for measuring $S_{C/O}$ (see next step).

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B. Rubisco purification for measuring $S_{C/O}$

The soluble cellular protein needs to be further purified before quantifying $S_{C/O}$ (CO_2/O_2 specificity).

1. Soluble cellular protein from step A3 above is manually passed by syringe through a 1 ml Bio-Scale mini Macro-Prep high Q ion exchange (IEX) column equilibrated with column buffer. All steps are performed at 4 °C. The method involves: Pre-washing the IEX column is with 2 ml of column buffer. Soluble cellular protein is passed through the column by syringe (flowrate at ~3 to 5 ml min^{-1}) and then the column is washed with 8 ml of column buffer. Bound Rubisco is eluted in 1.5 ml column elution buffer, collecting in three 500 µl elution fractions. The last two fractions contain > 90% of the Rubisco activity and are therefore pooled before concentrating to ~0.4 ml by centrifugation (4,000 *x g*, 10 min, 4 °C) using an Amicon Ultra-100 centrifugal filter (*i.e.,* a 100 kDa MW cut-off filter).
2. The concentrated Rubisco is injected into the 200 µl sample loop of the Äkta purification system onto a Superdex 200 column pre-equilibrated with $S_{C/O}$ buffer at 4 °C. The sample is loaded onto the column using the flow rate of 0.5 ml/min (max delta column pressure alarm set to 1.5 MPA) and fractions (0.5 ml) collected after the void volume (8 ml). Absorbance at 280 nm and conductance are continuously monitored. RuBP dependent $^{14}CO_2$-fixation activity in the fractions is assayed and those with peak Rubisco activity (typically fractions 5 to 7) are pooled and concentrated to ~0.1 ml by centrifugation (4,000 *x g*, 20 min, 4 °C) using an Amicon Ultra-100 centrifugal filter.
3. The concentrated and purified Rubisco is now ready for the $S_{C/O}$ assay. Alternatively, glycerol can be added to 20% (v/v) final concentration and the enzyme then frozen in liquid nitrogen and stored at -80 °C.
4. Optional: the $S_{C/O}$ assay (not part of this protocol on Rubisco extraction)
   a. $S_{C/O}$ assays are carried out at 25 °C (or other temperature) according to the method of (Kane *et al.*, 1994). The purified Rubisco (10-50 µl) is injected into 20 ml glass, septum sealed glass vials containing 1 ml assay buffer (see Recipes) that has been equilibrated for 0.5 to 1 h with 500 ppm CO_2 mixed with O_2 using Wostoff gas-mixing pumps. After 15 min, further equilibration the reactions are initiated with the injection of 1 nmol of 2-^{3}H-RuBP. Alkaline phosphatase is injected after 30 min to convert the 3-^{3}H-phosphoglycerate (carboxylation product) and 2-^{3}H-phosphoglycolate (oxygenation product) into ^{3}H-3-glycerate and ^{3}H-2-glycolate.
   b. The reactions are passed through a 0.4 ml AGI-X8 (10% formate) anion-exchange column and then the bound ^{3}H-3-glycerate and ^{3}H-2-glycolate eluted in 0.5 ml 20% (v/v) H_2SO_4 before separating them by HPLC using an isocratic gradient of 0.012 M H_2SO_4 through a Aminex HPX-87H column at 65 °C at a flow rate of 0.4 ml min^{-1}.
   c. The $S_{C/O}$ at 25 °C is then calculated from the radioactivity in the ^{3}H-3-glycerate and ^{3}H-2-glycolate using equation:

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$$S_{C/O} = \frac{[glycerate]}{[glycolate]} \times \frac{M[O_2]}{M[CO_2]} \times 0.037$$

Where,

$\frac{M[O_2]}{M[CO_2]}$ represents the molar ratio of O_2 and CO_2 (99.95% and 0.05% [v/v] respectively), 0.037 is the proportion of the CO_2 solubility relative to O_2 in H_2O at 25 °C (Kane *et al.*, 1994).

Data analysis

1. Intact Rubisco remains in the soluble fraction during extraction
   It was determined that full cell lysis is routinely obtained using a French Pressure cell and that Rubisco remains intact and within the soluble fraction during the crude extraction (steps A1-A3). Native PAGE (Figure 2) shows Rubisco remains as an intact L_8S_8 enzyme (comprising 8 large [L] and 8 small [S] subunits), even following electrophoresis for 16 h at 4 °C and 60 V. The differing mobility of the Rubsico band between species occurs due to subunit sequence differences and subtle variations in quaternary structural properties (*i.e.*, slight differences in amino acid sequence and length). SDS-PAGE (Figure 3) demonstrates that comparable levels of Rubisco L- and S-subunit are detected in the whole cell lysis (after mechanical rupture but prior to centrifugation, step A2) and soluble cell protein (after centrifugation, step A3) indicating the extracted Rubisco is fully soluble.
2. Rubisco retains activity after extraction
   The activity of Rubisco within the crude extracts (steps A1-A3) of 11 diatom species were tested and published (Young *et al.*, 2016). Full activation of Rubisco was obtained after 10 min of extraction at 25 °C and the activity remained stable during a further 10 min testing. Measurements of K_C and k_{cat}^c for the diatom Rubiscos are published in (Young *et al.*, 2016).
3. Quantifying $S_{C/O}$ (steps B1-B4). Figure 4 shows the ^{3}H-elution profile of the HPLC separated ^{3}H-glycerate and ^{3}H-glycolate peaks which are used to calculate $S_{C/O}$ as described above in step B4c.

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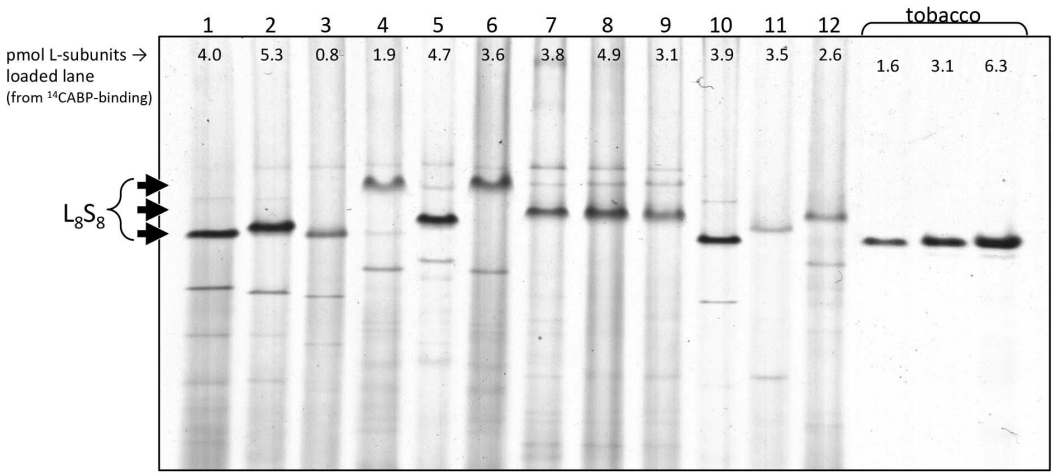

Figure 2. Native-PAGE blot of crude extract to show fully intact Rubisco protein. Total soluble cell extract (5 µg as determined by Bradford assay, Coomassie plus) was separated at 4 °C through a precast 4-12% Tris-glycine gel overnight (16 h) at 60 V. Gel was rinsed with deionized water, fixed for 30 min with 45% (v/v) H_2O, 5% (v/v) acetic acid and 50% (v/v) methanol the extensively rinsed with multiple changes if deionized water. The proteins were visualized using Gelcode blue Coomassie stain (Invitrogen). Arrows indicate where the ~520 kDa Rubisco complex (L_8S_8) locates on the gel. Lane numbers indicate extract from different diatom species: (1) *Thalassiosira weissflogii* CCMP 1336, (2) *Thalassiosira oceania* CS-427, (3) *Skeletonema marinoi* CCMP 1332, (4) *Chaetoceros calcitrans* CCMP 1315, (5) *Chaetoceros calcitrans* CS-178, (6) *Chaetoceros muelleri* CCMP 1316, (7) *Phaeodactylum tricornutum* CCMP 642, (8) *Phaeodactylum tricornutum* UTEX 630, (9) *Phaeodactylum tricornutum* CS-29, (10) *Bellerochea* sp. CS-874/01, (11) *Isochrysis* sp. CS-177, (12) *Pleurochrysis cartera* CS-287. Last three lanes contain 5, 10 and 20 µl of crude cell extract from tobacco as a control. Shown are the Rubisco active site contents quantified for each sample by ^{14}C-CABP binding (Whitney and Sharwood, 2014; Sharwood *et al.*, 2008).

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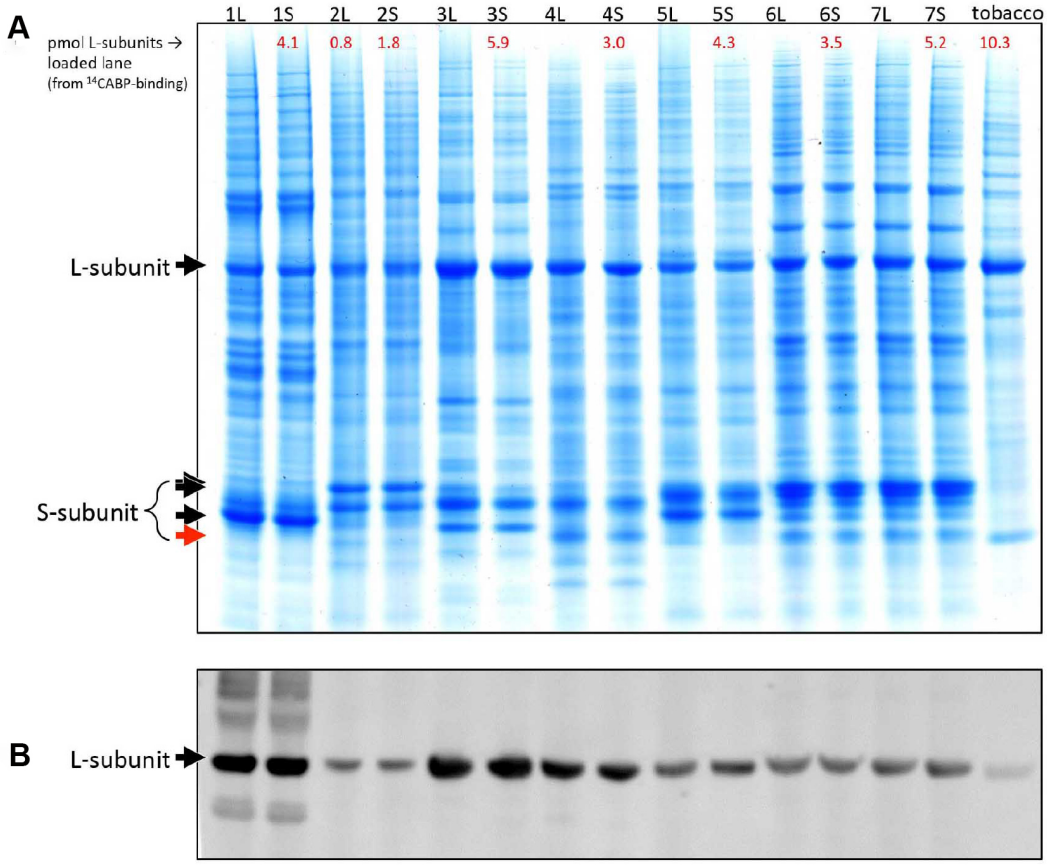

Figure 3. SDS-PAGE analysis of Rubisco solubility, integrity and complete extraction by French Pressure Cell lysis. A. Coomassie stain and B. Diatom Rubisco antibody blot (see Whitney *et al.*, 2001 for details) of total cellular lysate (L, following French Pressure Cell lysis) and soluble cellular protein (S, following centrifugation) from the diatom species: (1) *P. tricornutum* CS-29, (2) *Skeletonema ardens* CS-348, (3) *Pavlova lutheri* CS-182, (4) *Fragilariopsis cylindrus* CCMP 1102, (5) *Cylindrotheca fusiformis* CS-13, (6) *Thalassiosira oceania* CS-427, and (7) *Thalassiosira weissflogii* CCMP 1336. 5 µl of tobacco soluble leaf protein was loaded for comparison. The equal intensity of the L-subunit in both the L and S protein fractions indicate all the Rubisco was extracted (complete cell lysis) and fully soluble with no L-subunit degradation evident in the Western blot. Sample preparation and electrophoresis: protein (L or S) extracts (150 µl) were added to 4x SDS-reducing buffer (50 µl) and boiled for 5 min then centrifuged (16,000 *x g*, 5 min) before separating by 4-12% Bis-Tris SDS-PAGE at 200 V for 45 min in MES buffer (50% methanol, 40% H_2O and 10% glacial acetic acid). Duplicate gels were either (A) fixed and Coomassie stained (see Figure 2) or (B) the proteins transferred onto nitrocellulose membrane and probed with an antibody to *P. tricornutum* Rubisco (see Whitney *et al.,* 2001 for further experimental details).

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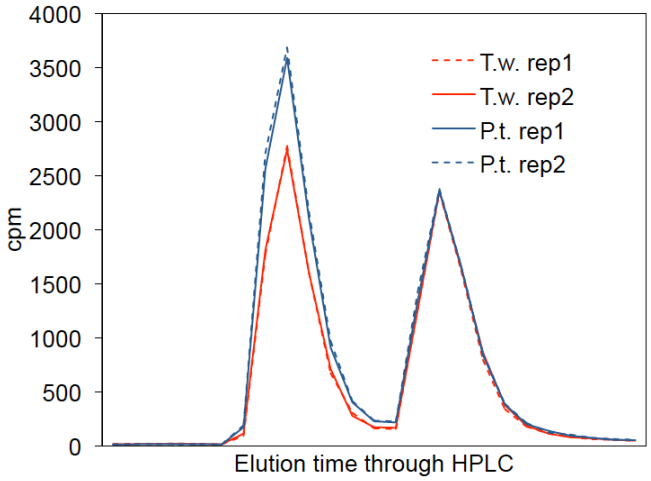

Figure 4. ^{3}H-Elution profile of the HPLC fractions that are used to calculate $S_{C/O}$ from the amount of ^{3}H incorporated into ^{3}H-glycerate and ^{3}H-glycolate (peaks 1 and 2 respectively) as described in step B4c. Shown here are results of an $S_{C/O}$ assay using purified Rubisco from the diatoms, *Thalassiosira weissflogii* (T.w., red) and *Phaeodactylum tricornutum* (P.t., blue) showing two technical replicates (rep) for each.

Recipes

1. Extraction buffer
   50 mM EPPS-NaOH, pH 8.0
   1 mM EDTA
   2 mM dithiothreitol (DTT)
   1% (v/v) plant protease inhibitor cocktail
   Dissolve EPPS and EDTA in deionized water to the final desired concentrations
   Bring pH to 8.0 with NaOH
   Notes:
   a. *Add DTT just prior to starting extractions.*
   b. *Immediately prior to use, add protease inhibitor cocktail to the aliquot of buffer being used for extraction.*
2. Column buffer
   50 mM EPPS-NaOH, pH 8.0
   1 mM EDTA
   10 mM NaCl
   Dissolve all salts in deionized water to the final desired concentrations
   Bring pH to 8.0 with NaOH
3. Column elution buffer
   50 mM EPPS-NaOH, pH 8.0
   1mM EDTA

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0.8 M NaCl
Dissolve all salts in deionized water to the final desired concentrations
Bring pH to 8.0 with NaOH

4. Specificity ($S_{C/O}$) buffer
   30 mM triethanolamine
   15 mM magnesium acetate, pH 8.3
   Dissolve all salts in deionized water to the final desired concentrations
   Bring pH to 8.3 with acetic acid
   Store at 4 °C
5. Assay buffer (1 ml)
   30 mM triethanolamine
   15 mM magnesium acetate, pH 8.3
   10 µg ml^{-1} carbonic anhydrase
6. 4x SDS reducing buffer for SDS-PAGE (Optional)
   125 mM Tris-HCl pH 6.8
   4% (w/v) SDS
   0.01% (w/v) bromophenol blue
   20% (v/v) glycerol
   75 mM 2-mercaptoethanol
   Dissolve all salts in deionized water to the final desired concentrations
   Add glycerol and 2-mercaptoethanol before use
7. BS buffer for SDS-PAGE (Optional)
   10 mM Tris-HCl, pH 7.5
   150 mM NaCl
   Dissolve all salts in deionized water to the final desired concentrations

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