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Extraction and Activity of O-acetylserine(thiol)lyase (OASTL) from Microalga *Chlorella sorokiniana*

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[Abstract] O-acetylserine(thiol)lyase (OASTL) is an enzyme catalysing the reaction of inorganic sulphide with O-acetylserine to form the S-containing amino acid L-cysteine. Here we describe an improved protocol to evaluate the activity of this enzyme from the microalga *Chlorella sorokiniana*. It is a colorimetric assay based on the reaction between cysteine, the product of OASTL activity, and ninhydrin reagent, which forms a thiazolidine (Thz).

Keywords: *Chlorella sorokiniana*, Colorimetric assay, Cysteine, Microalgae, Ninhydrin, O-acetylserine(thiol)lyase, Sulphur

[Background] In archea, bacteria, microalgae and plants, the synthesis of cysteine (Cys) represents a decisive stage of assimilatory sulphate reduction (Hell and Wirtz, 2008). Cys biosynthesis is the last step of sulphur assimilation and proceeds by two interconnected reactions catalysed by serine acetyltransferase (SAT, EC 2.3.1.30) and O-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8) (Salbitani *et al*., 2014; Carfagna *et al*., 2015).

OASTLs catalyse the reaction between O-acetilserine (OAS) and sulphide to form Cys and acetate (Figure 1).

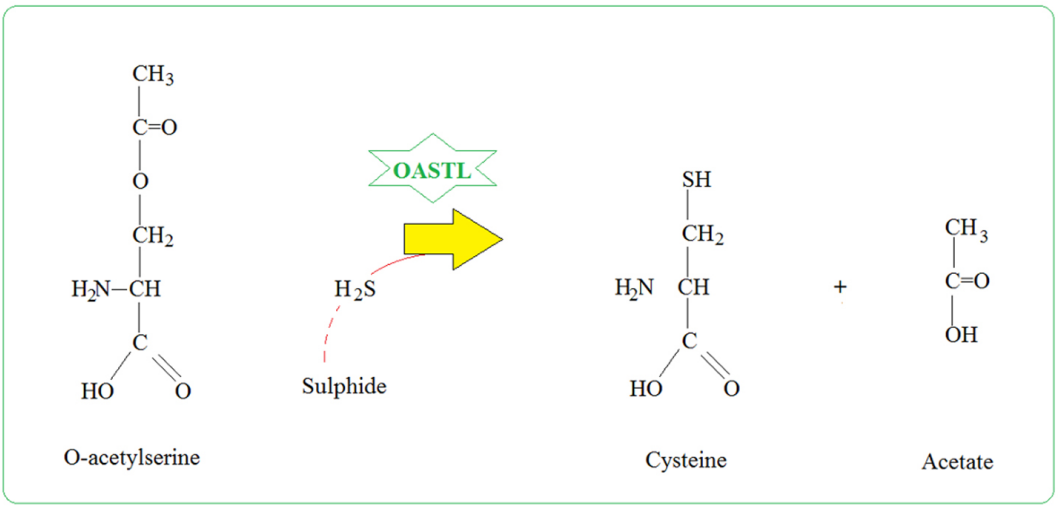

Figure 1. Schematic mechanism of cysteine biosynthesis catalyzed by O-acetylserine(thiol)lyase

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In vascular plants, OASTLs are localized in chloroplasts, mitochondria and the cytosol with different functions for Cys synthesis (Jost *et al*., 2000; Birke *et al*., 2013). In microalgae, OASTLs seem to be mainly localized essentially in the chloroplasts (Merchant *et al*., 2007; Bromke, 2013). However, in *Chlorella sorokiniana* two isoforms, chloroplastic and cytosolic OASTL, were found under S-deprivation conditions (Carfagna *et al*., 2011).

Many researchers have developed and modified protocols to determine OASTLs activity in plants and bacteria (Gaitonde, 1967; Burnell and Whatley, 1977; Lèon *et al*., 1987; Rolland *et al*., 1992). Here we describe a protocol for the determination of OASTL activity, optimized for the green microalga *Chlorella sorokiniana* 211-8K (Figure 2). This OASTL assay is a spectrophotometric analysis based on the colorimetric reaction of the formed L-cysteine with ninhydrin reagent to form a thiazolidine (Thz) (Prota and Posiglione, 1973).

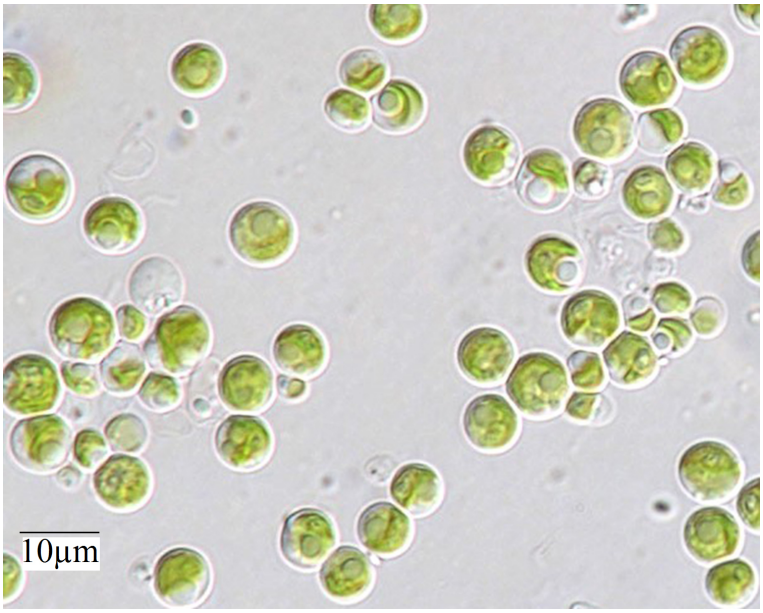

Figure 2. Optical microscope image of *Chlorella sorokiniana* cells

Materials and Reagents

1. Eppendorf tubes (1.5-2.0 ml)
2. Cuvettes 1.5 ml (BRAND, catalog number: 759115)
3. CO_2 tank
4. *Chlorella sorokininana* Shihira & Krauss, strain 211/8K (CCAP, Cambridge University) (Figure 2)
5. Liquid nitrogen
6. Milli-Q water
7. O-Acety-lserine (OAS) (Sigma-Aldrich, catalog number: CDS020792)
8. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D9779)
9. Sodium sulfide nonahydrate ($Na_2S{\cdot}9H_2O$) (Sigma-Aldrich, catalog number: S2006)

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10. Trichloracetic acid (TCA) (Sigma-Aldrich, catalog number: 91228)
11. Acetic acid (CH_3COOH) (Avantor Performance Materials, J.T. Baker®, catalog number: 401424)
12. Ethanol (EtOH) (Avantor Performance Materials, J.T. Baker®, catalog number: 8007)
13. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, catalog number: 5000006)
14. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
15. Potassium phosphate dibasic (K_2HPO_4) (Sigma-Aldrich, catalog number: 04248)
    Note: This product has been discontinued.
16. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S5886)
17. Magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, catalog number: M2643)
18. Ethylenediaminetetraacetic acid ferric sodium salt (Fe-EDTA) (Sigma-Aldrich, catalog number: E6760)
19. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C5670)
20. Potassium nitrate (KNO_3) (Sigma-Aldrich, catalog number: P8291)
21. Copper(II) sulfate ($CuSO_4$) (Sigma-Aldrich, catalog number: 451657)
22. Ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}·4H_2O$ (Sigma-Aldrich, catalog number: M1019)
23. Manganese(II) chloride ($MnCl_2$) (Sigma-Aldrich, catalog number: 13217)
    Note: This product has been discontinued.
24. Zinc sulfate heptahydrate ($ZnSO_4·7H_2O$) (Sigma-Aldrich, catalog number: Z0251)
25. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B6768)
26. Pyridoxal-phosphate (PLP) (Sigma-Aldrich, catalog number: P3657)
27. Ninhydrin (Sigma-Aldrich, catalog number: N4876)
28. Hydrochloric acid 37% (Avantor Performance Materials, J.T. Baker®, catalog number: 6012)
29. HEPES (Sigma-Aldrich, catalog number: H4034)
30. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A7030)
31. Basal medium (see Recipes)
32. Phosphate buffer (see Recipes)
33. Extraction buffer (see Recipes)
34. Ninhydrin solution (see Recipes)
35. 1 M HEPES solution (see Recipes)

Equipment

1. Culture flask (WHEATON, catalog number: 356954)
2. Fluorescent lamps (Philips Lighting, model: TL-D 30W/55)
3. Bench centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: IEC CL30)
4. French pressure cell press (AMINCO RESOURCES, model: FA-078)

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5. Superspeed centrifuge (Thermo Fisher Scientific, Thermo Scientific™, model: Sorvall RC-5C Plus)
6. Vortex mixer (Troemner, catalog number: 945302)
7. Eppendorf ThermoMixer® Comfort (Eppendorf, model: 5355)
8. Eppendorf MiniSpin® (Eppendorf, model: 5453000011)
9. Thermo bath (Labortechnik medingen, model: MWB 5)
10. Spectrophotometer (Cole-Parmer, JENWAY, model: 7315)
11. Optical microscope (Esselte, Leitz, model: Leitz Laborlux K)

Software

1. SigmaPlot® 12 software

Procedure

A. Algal culture condition

Grow *Chlorella sorokiniana* culture in batch in basal medium (see Recipes) at 35 °C and under continuous illumination (fluorescent lamps, 250 µmol photons m^{-2} sec^{-1}). Bubble the culture with air containing 5% CO_2. Under these conditions, the algal growth rate constant (µ) is 3 d^{-1}.

B. Preparation of microalgae extracts

1. Harvest 200 ml of algal culture by centrifugation (4,500 *x g* for 10 min). Collect the cells during the exponential growth phase (culture OD_{550} between 0.5 and 1.0).
2. Discard the supernatant and re-suspend the pellet in 5 ml of cold (4 °C) extraction buffer (see Recipes).
3. Lyse the cells by passing twice through a French pressure cell (1,100 psi).
   Note: Other methods and procedures can be used to lyse microalgae; among the most common methods, there are the use of magnetic stirrer, microwave radiation, ultrasonication and enzyme treatment (Dvoretskyet al., 2016; Farooq et al., 2016; Huang et al., 2016). The breaking of the cells can be observed with an optical microscope.
4. Centrifuge cell homogenate at 11,000 *x g* for 15 min at 4 °C. Use the resulting supernatant as crude extract (CE) and assay it for enzyme activity.
5. Keep the crude extract on ice or freeze the sample in liquid nitrogen and store at -80 °C for future assays.
   Note: Store the samples at -80 °C for a maximum period of six months. The frozen sample can be thawed at room temperature.

C. OASTL assay

1. Add the following solutions as shown in the Table 1 to Eppendorf tubes (1.5-2.0 ml)

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Table 1. OASTL protocol assay

	Stock solutions	Blank	Sample	Final concentration
1	Milli-Q water	65 µl	55-60 µl - CE µl	
2	1 M HEPES/KOH, pH 7.20	10 µl	10 µl	100 mM
3	100 mM OAS	10 µl	10 µl	10 mM
4	100 mM DTT	5 µl	5 µl	5 mM
5	CE		5-10 µl	
6	50 mM Na_2S	10 µl	10 µl	5 mM
7	Final volume	100 µl	100 µl	

Notes:

a. *For the preparation of the reaction mix, it is important to respect the order of the solutions as shown in the Table 1.*
b. *The volume of Milli-Q water in the sample depends on the amount of CE used, considering a final volume of the reaction mix of 100 µl.*
c. *OAS (O-acetylserine) needs to be prepared fresh before use. DTT and Na_2S can be prepared, aliquoted and stored at -20 °C for six months.*

2. After preparation of the reaction mix, vortex the samples and incubate the tubes in a thermomixer at 50 °C for 5 min.
3. Stop the reaction by adding 50 µl of 20% (v/v) trichloracetic acid (TCA). Spin at 13,000 *x g* and transfer the supernatants to clean tubes.
   Note: TCA can be stored at 4 °C for six months.
4. Add to the tubes 100 µl of glacial acetic acid and 200 µl of ninhydrin solution (see Recipes).
5. Incubate the samples at 100 °C for 5 min and then cool them in a Thermo bath at 10 °C for 5 min.
   Note: As alternative to Thermo bath, the samples can be incubated in a thermomixer at 99 °C.
6. Add 550 µl of ethanol, vortex and read the absorbance spectrophotometrically at 560 nm.

Data analysis

1. To evaluate the range of reliable activity, it is necessary to make a calibration curve for L-cysteine using known concentrations of the amino acid (0.1-3.0 mM).
   Note: We estimated valid an absorbance range at 560 nm between 0.5-1.5.
2. Enzymatic units were calculated using the following formula:
   Enzymatic units (U) = $A_{560}V_2V_0/\varepsilon t_1V_1V_e$
   where, A_{560} is the absorbance at 560 nm; V_2 is the final volume (ml) including ethanol; V_0 is the volume of the reaction mix incubated at 50 °C; ε is the OAS molar extinction coefficient (mM^{-1} cm^{-1}); t_1 is the time (min) of incubation at 100 °C; V_1 is the volume of the reaction mix incubated at 50 °C included TCA; V_e is the crude extract volume used for the assay (ml).
   Note: We estimated ε = 6.4 mM^{-1} cm^{-1}.

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3. OASTL activity was expressed in units that correspond to the formation of 1 µmol of cysteine min^{-1}. The OASTL activity in each sample should be correlated with the soluble protein content (mg ml^{-1} extract) that was determined by the Bio-Rad Protein Assay based on the Bradford method (Bradford, 1976), using bovine serum albumin as the standard. The enzymatic unit correlated with the protein content is U mg^{-1} protein.
   Note: The use of other methods for protein determination is possible.
4. Data of the mean ± SE of 3-6 independent experiments should be presented (Figure 3).
5. Experimental data analyses and graphs could be carried out using SigmaPlot® 12 software (Carfagna *et al.*, 2016).
   Note: The use of other software to analyze data is possible.

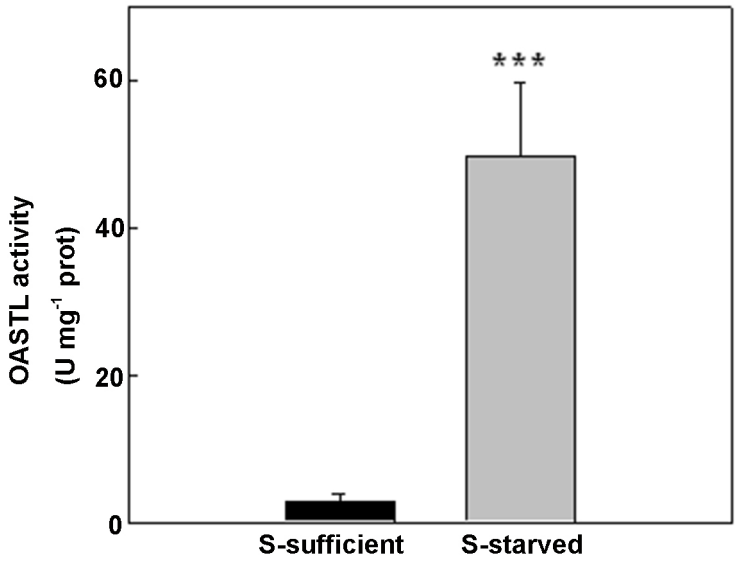

Figure 3. OASTL activity in *Chlorella sorokiniana*. Effect of 24 h sulphur-starvation on enzymatic activity (Salbitani *et al.*, 2014).

Recipes

1. Basal medium
   13 mM KH_2PO_4
   4.3 mM K_2HPO_4
   0.35 mM NaCl
   1.2 mM $MgSO_4$
   0.35 mM Fe-EDTA
   0.18 mM $CaCl_2$
   5 mM KNO_3
   Oligoelements: 0.31 mM $CuSO_4$, 0.12 mM $(NH_4)_6Mo_7O_{24}$, 14 mM $MnCl_2$, 0.76 mM $ZnSO_4$, 46 mM H_3BO_3
   Adjust the pH to 6.5 and sterilize by autoclaving. The medium is stored at 2-8 °C, in the dark

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2. Phosphate buffer, pH 7.5
   To prepare 50 mM phosphate buffer pH 7.5, mix 0.94 ml of 1 M KH_2PO_4 with 4.06 ml of 1 M K_2HPO_4 and add distilled water up to a final volume of 100 ml
   Note: Store the buffer at 2-8 °C for three months. The pH should be measured prior to use and adjusted, if necessary.
3. Extraction buffer
   50 mM phosphate buffer pH 7.5
   10 µM PLP (pyridoxal-phosphate)
   1 mM DTT (dithiothreitol)
   Note: Prepare fresh before use.
4. Ninhydrin solution
   To prepare the ninhydrin solution dissolve 0.12 g of ninhydrin in 3 ml of glacial acetic acid and 2 ml of hydrochloric acid 37%
   Note: The ninhydrin solution can be stored in the dark at room temperature for five days.
5. 1 M HEPES solution
   Dissolve 11.9 g of HEPES in Milli-Q water up to a final volume of 50 ml
   Adjust the pH to 7.20 with KOH
   Note: Store the buffer at 2-8 °C for three months. The pH should be measured prior to use and adjusted, if necessary.

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