Quantification of Low Molecular Weight Thiols in *Arabidopsis*

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[Abstract] Low-molecular-weight (LMW) thiols are a class of highly reactive compounds due to their thiol moiety. They play important roles in the maintenance of cellular redox homeostasis, detoxification, and development. Monobromobimane (mBBr) is weakly fluorescent but selectively reacts with thiols to yield highly fluorescent thioethers (mBSR) products, which is especially useful for the quantification of LMW thiols. The stable mBSR products can be separated by high-performance liquid chromatography (HPLC) equipped with a fluorescent detector. The main cellular LMW thiols are L-cysteine, gamma-glutamylcysteine, and glutathione (GSH). The following protocol describes the extraction and quantification of L-cysteine, gamma-glutamylcysteine, and glutathione from *Arabidopsis* tissues as reported (Xiang and Oliver, 1998; Zhao *et al*., 2014; Wang *et al*., 2015) with minor revision. Modifications may be required if the HPLC system or the C18 column is different.

Materials and Reagents

1. 1.5 ml microcentrifuge tube
2. 0.2 μm nylon filter (Sigma-Aldrich, catalog number: Z259969)
3. 100 ml glass syringe
4. Pipette tip
5. Fresh *Arabidopsis thaliana* tissues (any plant part you want to test, 50-100 mg is sufficient)
6. L-cysteine (Sigma-Aldrich, catalog number: 778672)
7. Gamma-glutamylcysteine (γ-Glu-Cys) (Sigma-Aldrich, catalog number: G0903)
8. L-Glutathione reduced (Sigma-Aldrich, catalog number: G4251)
9. Double distilled water (supplied by School of Life Sciences, University of Science and Technology of China)
10. Double distilled water
11. Hydrochloric Acid (HCl) (Sigma-Aldrich, catalog number: 435570) (see Recipes)
12. 2-(N-Morpholino)ethanesulfonic acid hydrate (MES) (Sigma-Aldrich catalog number: M8250) (see Recipes)
13. Ethylenediaminetetraacetic acid disodium salt dihydrate ($EDTA{\cdot}2Na{\cdot}2H_2O$) (Sangon Biotech, catalog number: A100105) (see Recipes)

14. Monobromobimane (mBBr) (Sigma-Aldrich, catalog number: 69898) (see Recipes)
15. Trifluoroacetic acid (TFA) (Sigma-Aldrich, catalog number: 302031) (see Recipes)
16. Acetonitrile (OCEANPAK, catalog number: Ac00030281) (see Recipes)

Equipment

1. Analytic balance (Mettler-Toledo International Inc., model: ML104)
2. Mortar and Pestle
3. Refrigerated microcentrifuge (Thermo Fisher Scientific, Eppendorf, model: 5424R)
4. 37 °C degree incubator (Shanghai Jinghong Laboratory Instrument, model: GNP-9080)
5. Reverse-phase C18 column (5 µm, 110A, 150 x 4.6 mm) (Phenomenex, model: Gemini C18 column) or equivalent, guard column (Phenomenex, SecurityGuard Standard, model: AJ0-7597)
6. HPLC equipment (Agilent Technologies, model: 1200 series)
   a. Pump (Agilent Technologies, model: G1312A)
   b. Sampler (Agilent Technologies, model: G1328A)
   c. Column heater (Agilent Technologies, model: G1316A)
   d. FLD detector (Agilent Technologies, model: G1321A)
7. 200 µl pipette
8. pH meter (Thermo Fisher Scientific, Mettler Toledo, model: FE20–FiveEasy Plus™)
9. Ultrasonic cleaner (Shanghai Sonxi Ultrasonic Instrument, model: DS-2510DT)

Software

1. Suitable data collection and processing software (Agilent Technologies, model: 1200 ChemStation)
2. Standard curve plotting (Microsoft Excel)

Procedure

A. Preparation of samples and standards
   1. Fresh tissues are sampled into a 1.5 ml microcentrifuge tube and weighed with an analytical balance. 50-100 mg is sufficient for each biological sample.
   2. Samples are ground in the microcentrifuge tube with a mortar and a pestle with 2 volumes of 0.15 M HCl added by 200 µl pipette (for example, 100 mg tissue needs 200 µl).
   3. The homogenate is centrifuged at 12,000 *x g*, 4 °C, for 15 min and the supernatant is transferred into a new 1.5 ml microcentrifuge tube. This step can be repeated if necessary to remove insoluble substances.

4. Prepare 10 mM stock solutions of L-cysteine, gamma-glutamylcysteine, and glutathione, and then prepare the following standards: 0, 50, 100, 200, 500, 1,000 μM of L-cysteine, gamma-glutamylcysteine, and glutathione by diluting the stocks with 0.15 M HCl.
5. 100 μl of the supernatant from step A3 (or standards) is transferred to 1.5 ml microcentrifuge tubes containing 2 μl of 0.5 M EDTA, 2.6 μl of 300 mM mBBr in acetonitrile, and 100 μl of 1.75 M MES (pH 7.4). The mixture was incubated in the dark at 37 °C for 1 h to allow the derivatization reaction to completed.
6. The mixture is centrifuged as in step A3 for 5 min before quantification.

B. Quantification by HPLC (refer to Agilent 1200 HPLC ChemStation Operation)

Select “Edit entire method” from “Method” menu and set parameters according to the following text. For quantification, 50 μl sample from step A6 is injected into the sample chamber of the HPLC system and separated using a reverse-phase C18 column and a flow rate of 0.8 ml/min on an Agilent 1200 HPLC system. Solvents A and B are used to elute the fluorescent derivatives with the gradient shown in the table below. The fluorescent derivatives (mBSR) are detected using fluorescence detector with excitation wavelength at 260 nm and emission at wavelength 474 nm.

Table 1. HPLC elution program

Time (min)	A (%)	B (%)	Rate of flow (ml/min)
0.0	90	10	0.8
0.3	85	15	0.8
14.0	80	20	0.8
15.0	0	100	0.8
19.0	0	100	0.8
20.0	90	10	0.8
24.0	90	10	0.8

1. Analyze standards and samples using the HPLC program above with at least 3 replicates.
2. Peak areas are integrated using the ChemStation software.
   Select “Data analysis” from the “View” menu to enter the picture data analysis.
   Select “Load signal” from the “File” menu to select your data file.
   Select “Integrate” from the “Integration” menu, and then the data is integrated.

C. Calculations

1. Prepare standard curves by plotting the concentration (μM) of the standards (Y-axis) *vs* the peak areas (X-axis) and add a trendline.

2. Determine the peak area for each sample and determine concentration using the trendline. Concentration (μM) = a * peak area + b (a and b are already calculated in the trendline).

Recipes

1. 0.15 M HCl (stored at room temperature)
   1.27 ml HCl (37%)
   100 ml double distilled water
   Filtered through 0.2 μm nylon filter using 100 ml syringe
2. 1.75 M MES (pH 7.4) (stored at 4 °C)
   3.41 g MES
   10 ml double distilled water
   Adjust pH to 7.4 using NaOH and filter through 0.2 μm nylon filter using 100 ml syringe
3. 0.5 M EDTA·2Na·2H_2O (stored at room temperature)
   18.6 g EDTA·2Na·2H_2O
   Dissolved in double distilled water, pH adjusted to 8.0 with NaOH, and volume brought to100 ml
   Filtered through 0.2 μm nylon filter using 100 ml syringe
4. 300 mM monobromobimane (stored at -20 °C)
   0.025 g monobromobimane
   307.3 μl acetonitrile
   12,000 *x g*, 4 °C, 30min to remove insoluble substances
5. Solvent A: 0.1% (v/v) trifluoroacetic acid (HPLC grade) (freshly prepared)
   1 ml trifluoroacetic acid
   999 ml double distilled water
   Filtered through 0.2 μm nylon filter using 100 ml syringe
6. Solvent B: 90% (v/v) acetonitrile (HPLC grade) (freshly prepared)
   900 ml 100% acetonitrile
   100 ml 0.1% (v/v) trifluoroacetic acid
   Filtered through 0.2 μm nylon filter using 100 ml syringe
   Solvent A and Solvent B need degas in the ultrasonic cleaner for 30 min with loose lid
7. 10 mM L-cysteine (stored at -20 °C)
   0.0606 g L-cysteine
   50 ml 0.15 M HCl
8. 10 mM gamma-glutamylcysteine (stored at -20 °C)
   0.1251 g gamma-glutamylcysteine
   50 ml 0.15 M HCl
9. 10 mM glutathione (stored at -20 °C)
   0.1537 g glutathione

50 ml 0.15 M HCl

10. Standards (stored at -20 °C)

	0 µM	50 µM	100 µM	200 µM	500 µM	1,000 µM
10 mM	0 µl	5 µl	10 µl	20 µl	50 µl	100 µl
0.15 M HCl	1,000 µl	995 µl	990 µl	980 µl	950 µl	900 µl

Acknowledgments

This protocol was modified from previous work described by Fahey and Newton (1987).

References

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