Analysis of pheophorbide *a* and other intermediates of the chlorophyll metabolism in *Arabidopsis* leaves by HPLC

Ryouichi Tanaka
Inst Low Temp Sci, Hokkaido Univ, N19 W8, Kita-ku, Sapporo, 060-0819, JAPAN
rtanaka@lowtem.hokudai.ac.jp

Summary

This article provides a quick guide for the analysis of various intermediates of the chlorophyll metabolism by HPLC. The method is based on that developed by Zapata et al (Zapata et al., 2000) which uses a C8 column for the separation of chlorophylls and carotenoids. Our lab protocol incorporates a slight modification of the original method for the analysis of various chlorophyll intermediates in the leaves of higher plants.

Introduction

Chlorophyll is synthesized from glutamate and degraded to several different monopyrrole molecules in higher plants (Hortensteiner, 2006; Tanaka and Tanaka, 2007). Some of the intermediates of chlorophyll metabolism play essential and multiple roles in various aspects of the cellular activities in higher plants. Readers may refer to the recent review articles on the biosynthetic and degradation pathways for chlorophyll and on the roles of the tetrapyrroles in plants (Hortensteiner, 2006; Nott et al., 2006; Tanaka and Tanaka, 2007). In this article, I describe an HPLC method used in our laboratory to analyze chlorophyll precursors and catabolites. We slightly modified the method originally developed by Zapata et al (Zapata et al, 2000) in order to shorten the overall analysis time without sacrificing the resolution. The original article explained that several carotenoid species can be separated by the method described; we, however, haven't yet determined if this method can be used for the accurate quantification of carotenoids. Specifically, we suspected that some portions of violaxanthin and neoxanthin could be isomerized. For an HPLC analysis, experience in dealing with an HPLC system may be required. Researchers who have never learned basic HPLC techniques may wish to consult with more experienced colleagues. A Japanese version of this guide is available on http://www.lowtem.hokudai.ac.jp/plantadapt/ayumi/.

Materials

Standard pigments may be required for quantification purposes. Below is a list of possible sources for several different pigments.

Chlorophyll *a* and *b*: Juntec Co. Ltd, Odawara, Japan

Hydroxymethyl chlorophyll *a*: Prepared from chlorophyll *b* according to the method of Ito et al., (Ito et al., 1996).

Pheophorbide *a*: Wako Chemicals, Tokyo, Japan

Protoporphyrin IX, Mg-Proto IX, Mg-Proto IX monomethylester: Frontier Scientific, UT, USA, http://www.frontiersci.com/

Carotenoids: DHI, Hørsholm, Denmark, http://www.dhigroup.com/

The absorption coefficients of various photosynthetic pigments are summarized in the reference by Rebeiz (2002).

An HPLC system with which two solvents can be run is required (Tanaka et al., 2003; Nagata et al., 2005).

Column: Symmetry C8 (150 x 4.6 mm, 3.5 μm particle size. Waters)

Pump and mixer (A low pressure gradient system is sufficient.)

Detector: Choose either a UV-VIS detector, a photodiode array (PDA) detector, or a fluorescent detector. A PDA detector is the best choice for identifying pigments, while a fluorescent detector is the best option for sensitivity. UV-VIS detectors are the most economical.

Guard column: Phenomenex AJO-4290 C8 4.0 L mm x 3.0 ID mm with a guard column holder, Phenomenex KJO-4282

For grinding many leaves at a time, we use a special grinding apparatus known as a ShakeMaster (BioMedical Science Co. Ltd., Tokyo, Japan). For the extraction of pigments from a few samples we use a glass homogenizer available from AsOne, Tokyo, Japan, or simply a pestle and mortar.

Extraction of pigments

(1) Weigh and grind leaves in acetone using a ShakeMaster grinding apparatus (BioMedical Science Co. Ltd., Tokyo, Japan).

Note: We typically use more than 200 μl of acetone to extract pigments from 10-20 mg of fresh or frozen leaves. It is important to use a sufficient amount of acetone because if an insufficient amount of acetone is used a substantial portion of chlorophyll will be converted to chlorophyllide by the action of chlorophyllase present in leaves.
After extraction, minimize exposure of extracts to light.

(2) Centrifuge extracts for 5 min at 10,000 *g*.

(3) Collect supernatant in a new tube. Repeat extraction with acetone and combine the supernatants.

(4) Add water so that the final concentration of acetone is 80%.
Note: A portion of the chlorophyll will be gradually epimerazed in acetone. Roughly speaking, 10% of chlorophyll *a* could be modified to form a chlorophyll *a* allomer within 24 h, which compound typically comes over a minute earlier than chlorophyll *a* in the HPLC system described in this guide. Moreover, the majority of β-carotene may be precipitated within 24 h.

(5) Load an aliquot of extracts immediately to the HPLC system.

HPLC analysis

(1) You can load an aliquot from your samples directly to the HPLC system. The injection volume is typically from 10 μl to 200 μl, depending on the maximum volume that can be injected to your HPLC system.

(2) We use the following solvents:

A: Methanol:Acetonitrile:0.25M aqueous pyridine = 50:25:25 (v:v:v)
B: Methanol:acetonitrile:acetone = 20:60:20 (v:v:v)

(3) The flow rate is 1.2 ml/min. The time program is as follows:

min	% (B Sol)
0	0

12	45
17	98
22.5	98
0	0

(4) Pigments are identified by their spectral patterns and/or co-injection with standards. Note: Chlorophyllide *a* and Mg-ProtoIX are eluted at the same time. These pigments should be identified with a photodiode array detector.

(5) Pigments are quantified by the integrated area of each pigment at a certain wavelength according to the calibration curve made with standard pigments. Most photosynthetic pigments including carotenoids can be detected at 410 nm. Chlorophyll precursors can be specifically monitored at 650 nm. For fluorometric detection of Mg-ProtoIX and Mg-ProtoIX ME, we use an excitation at 417 nm and detect fluorescence at 600 nm.

References

Hortensteiner S (2006) Chlorophyll degradation during senescence. Annu Rev Plant Biol 57**:** 55-77

Ito H, Ohtsuka T, Tanaka A (1996) Conversion of chlorophyll b to chlorophyll a via 7-hydroxymethyl chlorophyll. J Biol Chem 271**:** 1475-1479

Nagata N, Tanaka R, Satoh S, Tanaka A (2005) Identification of a Vinyl Reductase Gene for Chlorophyll Synthesis in Arabidopsis thaliana and Implications for the Evolution of Prochlorococcus Species. Plant Cell 17**:** 233-240

Nott A, Jung HS, Koussevitzky S, Chory J (2006) Plastid-to-nucleus retrograde signaling. Annu Rev Plant Biol 57**:** 739-759

Rebeiz CA (2002) Analysis of intermediates and end products of the chlorohyll biosynthetic pathway. *In* AG Smith, M Witty, eds, Heme, Chlorophyll, and Bilins: Methods and Protocols. Humana Press, NJ, pp 111-155

Tanaka R, Hirashima M, Satoh S, Tanaka A (2003) The Arabidopsis-accelerated cell death gene ACD1 is involved in oxygenation of pheophorbide a: Inhibition of the pheophorbide a oxygenase activity does not lead to the "stay-green" phenotype in Arabidopsis. Plant & Cell Physiology 44**:** 1266-1274

Tanaka R, Tanaka A (2007) Tetrapyrrole biosynthesis in higher plants. Annu Rev Plant Biol 58**:** 321-346

Zapata M, Rodriguez F, Garrido JL (2000) Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C-8 column and pyridine-containing mobile phases. Marine Ecology-Progress Series 195**:** 29-45