High Performance Liquid Chromatography of Pheophorbide a and Pyropheophorbide a in Salted Vegetables and Chlorella

(Received August 27, 1984)

Yuiko TAKEDA, Sadao UCHIYAMA and Yukio SAITO

(National Institute of Hygienic Sciences: 1-18-1,
Kamiyoga Setagaya-ku, Tokyo, Japan)

The photosensitizers pheophorbide a and pyropheophorbide a, derived from chlorophyll in salted green vegetables and chlorella, were determined by high performance liquid chromatography under the following operating conditions: μ -Bondapak C-18 reverse-phase separation with methanol-2% phosphate solution (90:10 v/v) as the mobile phase, and UV detection at 415 nm. A flow rate of 1.0 ml/min at room temperature gave a good base line separation of pheophorbide a and pyropheophorbide a (retention times of 12.1 and 14.8 min, respectively).

The method was applied to the rapid monitoring of poisonous chlorophyll derivatives in vegetables and chlorella. Pheophorbide a was detected in fresh vegetables, but pyropheophorbide a was not. In salted vegetables stored for a long time (more than 3 months), almost all the chlorophyll had been converted to pyropheophorbide a. In commercial chlorella, pheophorbide a was detected to a far greater extent than pyropheophorbide a.

Key words: pheophorbide a; pyropheophorbide a; salted vegetable; chlorella

Introduction

Some of the decomposition products of chlorophyll, pheophorbide a and pyropheophorbide a, are known to be causal substances of food intoxication⁽¹⁻⁵⁾. The former is derived from chlorophyll by the action of chlorophyllase in plants and bacteria under acidic conditions, while the latter is derived from the former (that is, pheophorbide a lost the methyl carboxyl group at C-10).

Many analytical methods for chlorophylls and their derivatives with biological activity on the human body have been proposed,⁽⁶⁻¹²⁾ but they are rather time consuming. In this work, we have devised a convenient and reliable high performance liquid chromatographic method which can be used to monitor the appearance of toxic pheophorbide a and pyropheophorbide a in chlorella and vegetables.

Experimental

1. Materials

Salted vegetables and chlorella samples were purchased from a market in Tokyo.

2. Apparatus

The HPLC apparatus used consisted of a Varian Model 5000 liquid chromatograph (Varian Associates, Inc., U.S.A.) fitted with a Rheodyne injection valve (Rheodyne, Cotati, CA, U.S.A.) and a 20- μ l sample loop. The column was a 30 cm $\times$ 3.9 mm i.d. column prepacked with μ -Bondapak, C-18 of 10.0- μ m particle size (Waters Associates, U.S.A.). A Hitachi wavelength-tunable effluent monitor (Hitachi, Ltd., Japan) was operated at 415 nm. Chromatograms were recorded on a Hitachi 056 recorder. The absorption spectrum was determined with a Hitachi 556 spectrophotometer.

3. Preparation of pheophorbide a and pyropheophorbide a

Pure pheophorbide a and pyropheophorbide

Fig. 1. Absorption spectra of pheophorbide a and pyropheophorbide a in methanol-2% phosphate solution (90:10 v/v)

a were prepared from *Chlorella protochloides* and from TAKANA (*Brassica juncea* var. *integrifolia*)-zuke according to the methods of Tamura et al.⁵⁾ and Amano et al.,¹⁰⁾ respectively.

The concentrations of the standard solutions of pheophorbide a and pyropheophorbide a ranged from 10 µg/ml to 100 µg/ml.

4. Chromatographic conditions

The HPLC solvents were filtered through a 10 µ filter and degassed with helium before use. The mobile phase was methanol-2% phosphate solution (90:10 v/v) mixture and the flow rate was 1.0 ml/min at room temp. Calibration curves were drawn by plotting the peak area as the ordinate against the concentration on the abscissa.

5. Extraction procedure¹⁸⁾

Ground chlorella (100 mg; in the case of homogenized vegetables, 2 g) was extracted with 40 ml of 85% (v/v) acetone. The aqueous acetone extract was mixed with 40 ml of diethyl ether, and the ether extract was collected and washed three times with 40 ml of 3% sodium sulfate solution. The ether extract was reextracted twice with 20 ml and 10 ml of 17% hydrochloric acid, and the acid extract

was diluted with 150 ml of 5% sodium sulfate solution then extracted with 20 ml of diethyl ether, twice. The ether extract was dried over anhydrous sodium sulfate and concentrated to near dryness. The residue was dissolved in 1.0 ml of methanol, and 0.5 ml of the methanol sol. was transferred onto a Sep-Pak Silica Cartridge column (No. 51900, Waters Associates, Inc.) and eluted with 2.5 ml of methanol-2% phosphate solution (90:10 v/v) mixture. The eluate was injected into the HPLC column. Sample solutions were reconstituted with a suitable quantity of methanol, depending on the concentration required.

Results and discussion

The absorption spectra of pheophorbide a and pyropheophorbide a are shown in Fig. 1. The molar absorption coefficients of pheophorbide a are λ max. nm (ε) 415 (83900), 667 (41300) and those of pyropheophorbide a are λ max. nm (ε) 415 (75100), 667 (36950).

Fig. 2 shows high performance liquid chromatograms of a standard and extracts from salted vegetables and chlorella. The resolution is sufficient for precise quantitative ana-

Fig. 2. HPLC chromatograms of pheophorbide a and pyropheophorbide a in salted vegetables and chlorella

Table 1. Relation Between the Results of the HPLC and HPTLC Methods for Quantitative Analysis of Pheophorbide a and Pyropheophorbide a

Sample	Method	Pheophorbide a		Pyropheophorbide a	
		($\mu\text{g/g}$)	C.V. (%)	($\mu\text{g/g}$)	C.V. (%)
Salted vegetable 1* (leaf)	HPLC	78.3 ± 2.5	3.2	230.5 ± 7.0	3.0
	HPTLC	80.5 ± 7.2	8.9	235.8 ± 2.03	4.2
Salted vegetable 2** (leaf)	HPLC	18.5 ± 0.2	1.1	320.2 ± 17.9	5.6
	HPTLC	20.0 ± 1.7	8.5	353.7 ± 33.5	9.5
		(mg%)		(mg%)	
Chlorella 1	HPLC	73.5 ± 2.8	3.8	5.2 ± 0.2	3.8
	HPTLC	75.1 ± 6.5	13.3	tr.	—
Chlorella 2	HPLC	58.2 ± 3.0	5.1	5.9 ± 0.3	5.1
	HPTLC	61.0 ± 4.5	7.4	tr.	—
Chlorella 3	HPLC	175.4 ± 8.5	4.8	10.5 ± 0.4	3.8
	HPTLC	190.2 ± 15.3	8.0	1.3 ± 0.1	7.5

* *Brassica* s.p., NOZAWANA

** *Brassica juncea* var. *integrifolia*, TAKANA

Fig. 3. Correlation between the results of the HPLC and HPTLC methods for quantitative analysis of pheophorbide a

Fig. 4. Correlation between the results of the HPLC and HPTLC methods for quantitative analysis of pyropheophorbide a

Table 2. The Concentrations of Pheophorbide a and Pyropheophorbide a Found in Fresh and Salted Vegetables and Chlorella on the Market

Sample	Stored (days)	Part	Pheophorbide a			Pyropheophorbide a		
			Max.	Min.	Mean	Max.	Min.	Mean
TAKANA* (fresh) (n=3)	0	leaf	23.5	21.5	22.5	nd	nd	nd
		stem	6.5	5.3	5.9	nd	nd	nd
		whole	19.6	17.3	18.4	nd	nd	nd
TAKANA*-zuke (n=3)	20	leaf	88.9	18.5	51.4	320.2	80.5	182.0
		stem	7.9	3.4	5.3	4.0	2.0	2.8
		whole	43.5	15.0	26.2	163.5	37.9	82.7
TAKANA*-zuke (n=4)	90	leaf	nd	nd	nd	560.5	450.8	534.8
		stem	6.3	nd	5.5	35.3	17.5	28.3
		whole	3.8	nd	2.0	244.3	205.2	235.3
NOZAWANA**-zuke (n=5)	7	leaf	150.4	78.3	129.3	254.2	211.7	235.1
		stem	3.0	2.0	2.6	1.9	1.2	1.6
		whole	62.0	44.2	53.3	102.4	85.8	95.0
HIROSHIMANA***-zuke (n=5)	7	leaf	184.8	116.5	140.4	295.3	94.8	165.5
		stem	6.7	2.7	4.1	5.4	1.7	3.3
		whole	124.4	79.9	95.0	198.7	63.8	111.4
(unit: $\mu\text{g/g}$)								
Chlorella (n=5)	—		211.5	5.8	74.3	10.5	3.9	5.5
(unit: mg%)								

* *Brassica juncea* var. *integrifolia*, TAKANA

** *Brassica* s.p., NOZAWANA

*** *Brassica pekinensis* var. *HIROSHIMANA*

lysis of pheophorbide a and pyropheophorbide a in samples.

Calibration curves showed good linearity and reproducibility from 0 to 500 ng. The limits of determination for the pigments were 5 ng. The average recoveries from samples spiked with from 10 ppm to 10 mg% were over 82.5% and the within-run variability coefficients were less than 5.8% in 3 cases. Because of its simplicity and sensitivity, this method is the preferred method for monitoring of toxic chlorophyll degradative products.

A comparison of HPLC and high performance thin layer chromatography (HPTLC) analyses is shown in Table 1. The coefficient of variation (C.V.) was 4.20-13.30% (n=7) in the HPTLC method, whereas it was 1.08-5.18% in the HPLC method. As shown in Fig. 3 and Fig. 4, the concentrations of both pigments in samples obtained by the HPLC method were comparable with those found by the HPTLC method (pheophorbide a, $r=0.992$; pyropheophorbide a, $r=0.958$).

Table 2 shows the contents of pheophorbide a and pyropheophorbide a in fresh and salted vegetables and chlorella purchased in a market. Pyropheophorbide a was not detected in fresh vegetables, but it was detected in salted vegetables. When salted vegetables were stored for long time (more than 3

months), most of the chlorophylls were converted to pyropheophorbide a. In the chlorella, pheophorbide a was detected to a much greater extent than pyropheophorbide a.

References

- 1) Hashimoto, Y., Tsutsumi, J.: Food Hyg. Soc. Japan 4, 185~191 (1963).
- 2) Hashimoto, Y., Tsutsumi, J.: Agric. Biol. Chem. 28, 467~470 (1964).
- 3) Isobe, A., Kimura, S.: Nutrition & Food. 29, 221 (1976).
- 4) Kimura, S., Isobe, A.: Japan Lipid Peroxide Research., 2(1), 39~41 (1978).
- 5) Tamura, Y., Maki, T., Shimamura, Y., Nishigaki, S., Naoi, I.: J. Food Hyg. Soc. Japan. 20, 173~180 (1979).
- 6) Vernon, L. P.: Anal. Chem. 32, 1144~1150 (1960).
- 7) Jones, I. D., White, R. C., Gibbs, E.: J. Food Sci. 28, 431~439 (1963).
- 8) White, R. C., Jones, I. D., Gibbs, E., Butler, L. S.: J. Agric. Food Chem., 20, 773 (1972).
- 9) Sherma, J., Latta, M.: J. Chromatogr., 154, 73~75 (1978).
- 10) Amano, R., Ike, K., Uchiyama, M.: Food Sanitation Research. 28, 739~745 (1978).
- 11) Uchiyama, M.: *ibid.*, 31, 423~436 (1981).
- 12) Schwartz, S. J., Wool, S. L., Elbe, J. H.: J. Agric. Food Chem.: 29, 533~536 (1981).
- 13) Takeda, Y., Saito, Y., Uchiyama, M.: J. Chromatogr. 280, 188~193 (1983).