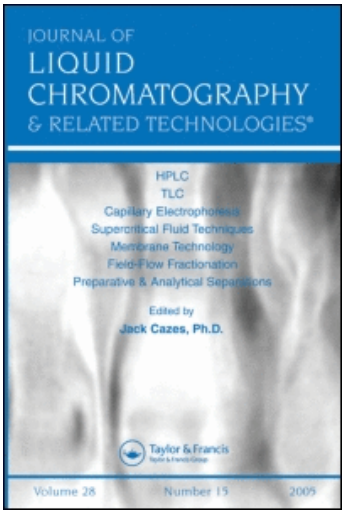

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Separation and Characterization of Chlorophyll Degradation Products in Silkworm Using HPLC-UV-APCI-MS

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ABSTRACT

By use of HPLC-UV-APCI-MS, the chemical nature of chlorophyll degradation products (CpD) in the excreta of the silkworm (*Bombyx mori*) was clarified in relation to its photosensitizing efficacy in photodynamic therapy (PDT) of tumors in vitro. Besides CpD from silkworm excreta aged in nature for more than a year, mulberry leaves, substances in the digestive system of silkworms fed on the mulberry leaves, and fresh excreta less than 24 hrs old were analyzed and compared. The CpDs isolated were separated and characterized, and their photosensitizing

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efficacy in experimental PDT was evaluated. Among the agents characterized by HPLC-UV-APCI-MS, pheophorbide-*a* and pyropheophorbide-*a* exerted the most potent photosensitizing effect in vitro. Although these pigments were detected in silkworm excreta, the amounts extracted were significantly higher in materials aged for more than one year. Yields from fresh excreta or digestive tract contents were lower than from stored excreta. None of those CpDs were detected in mulberry leaves. Natural environments presumably provide favorable conditions for the degradation of chlorophyll to pheophorbide-*a* and/or pyropheophobide-*a*.

Key Words: Chlorophyll degradation; Silkworm excreta; Pheophorbide-*a*; Pyropheophorbide-*a*; Photosensitizing effect; Photodynamic therapy (PDT); HPLC-MS.

INTRODUCTION

Photodynamic therapy (PDT) has been well known as a useful therapeutic measure for some cancers.[1] PDT involves the application of a photosensitizing agent and subsequent irradiation with light of a selected wavelength to activate the photosensitizer, which catalyzes the production of the reactive oxygen species to produce cytotoxicity.[2]

During a decade, search efforts have been focused on developing new sensitizers that can minimize side reactions and have stronger absorbance at 650–850 nm, since light of longer wavelengths penetrates farther into tissue. Therefore, new types of photosensitizers are undergoing clinical trials of PDT, including Photofrin®,[3] Purlytin (SnET2, tin etiopurpurin),[4] lutetium texaphyrin (Lu-tex),[5] benzoporphyrin derivative-monoacid ring A (BPD-MA),[6] and Npe6 (N-Aspartyl Chlorin).[7] These photosensitizers have been reported to be effective to in vitro. In pre-clinical studies, however, some drawbacks, including long term cutaneous photosensitivity or the need for high dose-light irradiation, were found in some studies.[6,7] Recently, pyropheophorbide-*a* methyl ester (PPME), a second generation photosensitizers, has been used in PDT.[8] Pyropheophorbide compounds have shown improved results as compared to drugs currently used in PDT. They facilitate tissue penetration by absorbing light of long wavelength (more than 660 nm) and are retained in the skin for short time periods compared to other drugs used in PDT.[9]

Our research group already have reported that a fraction isolated from silkworm excreta has successfully been used as a photosensitizer.[10,11] Since silkworms raised for commerce are fed only mulberry leaves, chlorin derivatives originating from the leaves were anticipated to be the agents responsible for the photodynamic reactions. The photosensitizing activity of chlorophyll degradation products (CpD) from silkworm excreta were also characterized.[12]

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Chlorophyll is an essential molecule of all plants. The natural catabolism of chlorophyll has been studied in senile leaves and ripe fruits of many plant species.[13–15] Spinach, algae, and fruit are commonly used for analyses of plant-pigment composition.[16–18] Pheophorbide-*a*, pyropheophorbide-*a*, and other derivatives of chlorophyll have been used as photosensitizers for PDT.[8,19,20] Although experimental PDT has been carried out successfully by using a fraction of silkworm excreta as a photosensitizer, the composition of silkworm excreta has not been previously reported.

In this study, we analyzed chlorophyll degradation products in silkworm (*Bombyx mori*) excreta, mulberry leaves, and substances in the digestive system of silkworms. Samples of fresh excreta collected within 24 hrs and the excreta aged for more than a year were studied to determine the types and amount of chlorophyll derivatives in them. By employing samples isolated and characterized as photosensitizers, experimental PDT was performed in vitro to compare their photosensitizing efficacies against human tumor cell line, HL-60.

EXPERIMENTAL

Sources of Chlorophyll Degradation Products

Mulberry leaves (*M. alba*, variety: Cheongilppong), live silkworms (*B. mori*, variety: Geumokjam), and fresh and stocked silkworm excreta were supplied by the Department of Agriculture and Entomology, National Institute of Agricultural Science and Technology, Suwon, Korea.

For extraction of chlorophyll degradation products from mulberry leaves, contents of the digestive system of dissected silkworm, and silkworm excreta, 1 g (powder) of each sample was thoroughly mixed with 20 mL acetone. The silkworm used was the fifth instar larva. Each sample was stirred by magnetic stirrer for 1 hr in an ice water bath in a dark room. The pigment solution was filtered through 0.45 μm membrane filters (Millipore, Bedford, MA, USA). The solvents were removed by use of a rotary evaporator. The dried pigments were dissolved (50 ppm) in the HPLC solvent A (75% acetonitrile/25% methanol), and then injected onto a high performance liquid chromatograph (HPLC) with an injector, Rheodine Model 7125 (Rohnert Park, CA, USA) equipped with a 20 μL sample loop. The temperature was maintained at 20°C, and stored at minus 78°C.

Measurement of Photosensitizing Effect by MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay[21] has been used successfully for PDT-mediated cytotoxicity measurement.

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In brief, HL-60 cells (1000 or 2500 cells per well) treated with chlorophyll degradation products from various sampling groups were plated in 100 μL RPMI in 96-well plates. A control medium contained the dimethylformamide vehicle. Cells were harvested 1 hr after the light irradiation. MTT activity was determined by adding 50 μL of MTT [5 mg/mL phosphate-buffered saline (PBS)] to 200 μL of medium in each well and incubating the mixture for 4 hrs at 37°C. Formazan crystals that formed within the attached cells were dissolved by adding 50 μL of dimethylsulfoxide to each well after the medium had been removed. Formazan production was measured at 570 nm on a 96-well microplate reader (Dynatec, Alexandria, CA, USA).

Chemicals

Samples of pure *β*-carotene, astaxanthin, echinenone, chlorophyll-*a*, and chlorophyll-*b* were purchased from Sigma (St. Louis, MO, USA). In general, analytical-reagent grade chemicals from J.T. Baker (Phillipsburg, NJ, USA) were used. For HPLC analyses, HPLC-grade solvents from Burdick & Jackson (Muskegon, MI, USA) were used. Eluents for HPLC were filtered through 0.45 μm membrane filters (Millipore, Bedford, MA, USA) and degassed before use.

HPLC Conditions for Isolation of CpD

An analytical separation by reversed-phase HPLC was performed on a Vydac 202TP54, C18 column (Hesperia, CA, USA), 5 μm, 300 Å, 4.6 × 250 mm. With Waters 600 (Milford, MA, USA) HPLC system, a Groton Technology (Acton, MA, USA) UV-VIS photodiode array detector was used. UV absorption was recorded at 430 nm. Pigments were eluted using a linear gradient in 20 min from 80% solvent A (75% acetonitrile/25% methanol) and 20% solvent B (0.01 M ammonium acetate in water) to 100% solvent A. The solution was maintained until the pigments were completely eluted. The flow rate was 1 mL/min with an analytical column.

Identification of CpD with HPLC-MS

Isolated pigments were identified according to their retention times, visible absorption characteristics, and mass spectra, as compared with references.[16–18] Each 20 μL sample solution was injected into a 4.6 × 250 mm Vydac 202TP54 column (Vydac Co., Columbia, MD, USA) for separation at 1 mL/min of flow rate using Waters 600 HPLC system and analyzed by

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HPLC-MS. The mass spectrometer was a VG Quattro triple quadrupole mass spectrometer (Fisons Instruments, VG Organic, Altrincham, UK) equipped with an atmospheric pressure chemical ionization (APCI) source. The API source was maintained at the following conditions: discharge voltage at 3 kV, nitrogen as a curtain gas, analyzer pressure at 1×10^{-6} mbar, and source pressure at 1×10^{-6} mbar. The corona discharge ion source was used with a heated nebulizer probe. The APCI source and probe temperature were maintained at 120°C and 450°C, respectively. Ions were introduced at atmospheric pressure into the mass analyzer via an intermediate pressure region defined by 20 V between sampling and skimmer plate. Data acquisition and instrument control were achieved by Mass Lynx (version 2.0) data system.

Photosensitizing Activity Assay

A human promyelocytic leukemia cell line, HL-60 (ATCC, Rockford, MD, USA) was cultured in RPMI-1640 (GIBCO-BRL), medium supplemented with 5 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO-BRL, Gaithersburg, MD) and 10% heat-inactivated fetal calf serum in a humidified atmosphere at 37°C with 5% CO_2. The chlorophyll degradation products from each sample group were added to cultures in 35-mm culture dishes to a final concentration of 20 μg 1 hr before light exposure, and then the cells were irradiated by the light. For all illuminations, the light source used was a 200 W halogen lamp (Micro Video Instruments Inc., Avon, MA, USA) attenuated by a 515 nm filter. The total power output for the light irradiation was adjusted to 120 mJ/cm^2 using a Laser power meter (Metrologic Instruments, Inc., Blackwood, NJ, USA).

Cells suspended in 100 μL of PBS were lysed by adding an equal volume of 2 × lysing buffer (200 mM HEPES, pH 7.5, 2% Triton X-100, 400 mM NaCl, and 20 mM EDTA). Following a 45 min digestion with 1 μg of RNase A at 37°C, samples were extracted with phenol/chloroform, precipitated with ethanol, and analyzed on a 2% agarose gel.

RESULTS AND DISCUSSION

PDT Effect of CpD

The basic structure of CpD is shown Fig. 1. First, we measured the PDT effect of extracts from mulberry leaves, substances in the digestive system of silkworm, and silkworm excreta against HL-60 tumor cells, respectively

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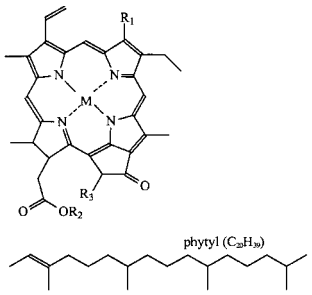

Pigments	M	R_1	R_2	R_3
Chlorophyll *a*	Mg	CH_3	$C_{20}H_{39}$	$COOCH_3$
Chlorophyll *b*	Mg	CHO	$C_{20}H_{39}$	$COOCH_3$
Chlorophyllide *a*	Mg	CH_3	H	$COOCH_3$
Chlorophyllide *b*	Mg	CHO	H	$COOCH_3$
Pheophytin *a*	2H	CH_3	$C_{20}H_{39}$	$COOCH_3$
Pheophytin *b*	2H	CHO	$C_{20}H_{39}$	$COOCH_3$
Pheophorbide *a*	2H	CH_3	H	$COOCH_3$
Pheophorbide *b*	2H	CHO	H	$COOCH_3$
Pyropheophorbide *a*	2H	CH_3	H	H
Pyropheophorbide *b*	2H	CHO	H	H

Figure 1. Basic structures of chlorophyll derivatives.

(Fig. 2). The extracts from silkworm excreta aged in nature for more than 1 year and from fresh excreta collected within 24 hrs, possessed a significant photosensitizing effect. The photosensitizing activity of the extracts from mulberry leaves and substances in the digestive systems of silkworm was negative or very weak.

Identification of CpD

To understand the photosensitizing effect of the silkworm excreta extracts, we investigated substances in the digestive system of silkworm. HPLC-UV-APCI-MS coupling was performed to separate and identify various chlorophyll-related compounds (Fig. 3). The identification of individual compounds was verified by HPLC-MS, and assignments were made by comparing their retention times, UV spectra, and mass spectra with those of known standards. Figure 3 shows the major components in mulberry leaves (3A), in substances in the digestive system of silkworm (3B), and in fresh silkworm excreta (3C), as determined by RP-HPLC.

Chlorophyll degradation occurred in the digestive system of the silkworms, as evidenced by the absence of chlorophyll-*a* in silkworm excreta [Fig. 3(C)], and the presence of chlorophyll degradation products such as chlorophyllide, pheophorbide, and pyropheophorbide. The general pathway of chlorophyll degradation we made (data not shown) is exactly coincident with other research.[22] Our results indicate that the digestive system of silkworm accelerate the degradation of chlorophyll and induce the formation of intermediated compounds in silkworm excreta.

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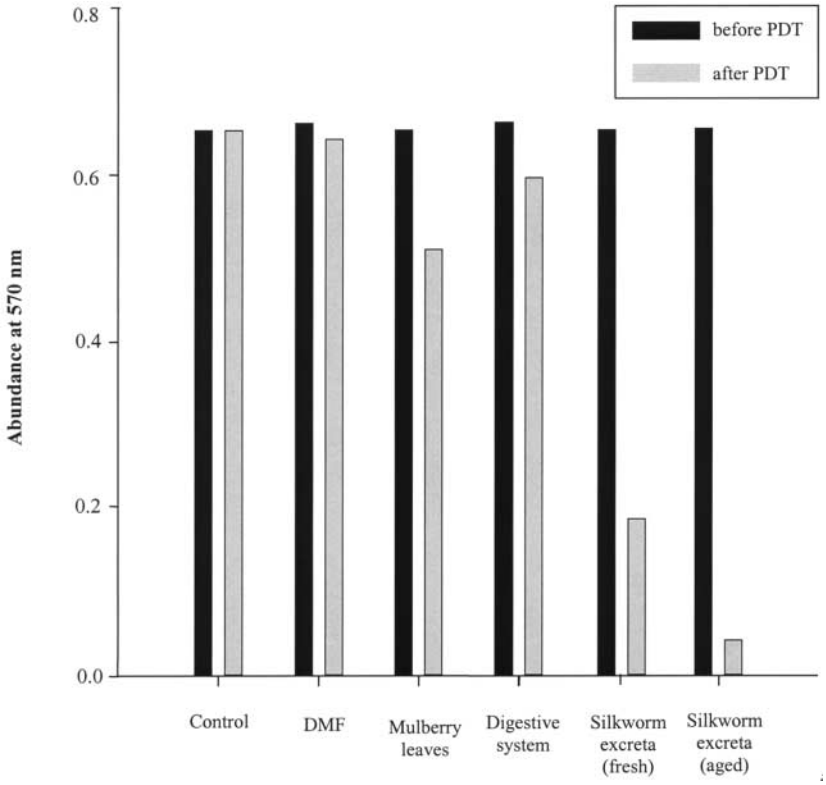

Figure 2. Cell death of HL-60 after PDT. Cells were incubated with concentrations (4 μg) of different photosensitizers for 60 min at 37°C. These were exposed to light (120 mJ) and were returned to the incubator. An MTT colorimetric assay was carried out 24 hours after PDT to assess cell survival.

Identification of the Pheophorbide-*a* and Pyropheophorbide-*a* from Silkworm Excreta

Among the substances from silkworm excreta separated by HPLC [Fig. 3(C)], components of peaks 3 and 5 exhibited a notable PDT effect against HL-60 cells (see below). These photosensitizing pigments were easily detected by their characteristic light absorption spectra, retention times [Fig. 4(A)], and positive-ion $[M+H]^+$ mass spectra [Fig. 4(B) and (C)].

The molecular weight of pheophorbide-*a*, which eluted at 8.0–8.2 min, is 592.1 Da. As shown in Fig. 4(B), the mass spectrum of pheophorbide-*a*

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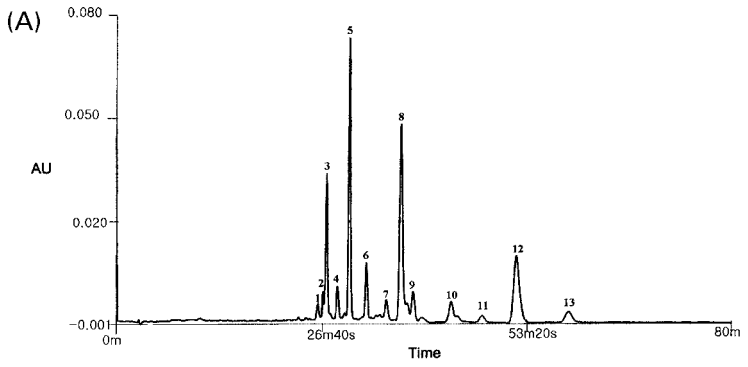
showed peaks at m/z 593, 535, and 518. The m/z 593 peak is the $[M+H]^+$ ion. The m/z 535 peak of mass spectrum was assumed to be formed by the removal of $COOCH_3$ from pheophorbide-*a* (MW 592.1), and the m/z 518 peak by the removal of $COOCH_3$ and CH_3 from pheophorbide-*a*. These fragmentation patterns were similar to the results obtained from standard pheophorbide-*a*.

Figure 4(C) shows the mass spectrum of pyropheophorbide-*a* (MW 534.1), which eluted at 12.7–14.1 min. The base peak at m/z 535, similar to the mass spectrum of pyropheophorbide-*a*, belonged to the protonated molecule, $[M+H]^+$ ion. The m/z 488 peak represented the removal of COOH from pyropheophorbide-*a*.



No	Pigment	Retention Time (min)	Absorbance maxima (nm)	Identification	$[M+H]^+$	Main fragment ions
1	Lutein	26.3	424, 449, 480	standard	551.5(100)	
2	Chlorophyll *b*'	26.4	459, 595, 648	UV		
3	Chlorophyll *b*	27.1	458, 595, 648	standard, MS, UV	907.5 (100)	629.1 (35)
4	Chlorophyll *b*'	28.3	458, 595, 648	UV		
5	Chlorophyll *a*	30.1	410, 426, 614, 665	standard, MS, UV	893.5 (100)	615.1 (16)
6	Chlorophyll *a*'	32.2	410, 426, 614, 665	MS, UV	893.5 (100)	615.1 (16)
7	IsoFxn-dhy Pde *a* ester	34.5	411, 469, 608, 665	UV		
8	Cryptoxanthin	36.5	448, 474	standard, MS, UV	552.1 (100)	
9	Pheophytin *b*	38.3	416, 441, 468, 578, 655	MS, UV	885.4(100)	607.3(25)
10	Unknown	43.2				
11	Pheophytin *a*	47.2	404, 500, 531, 604, 666	MS, UV	871.5 (100)	593.2 (13)
12	Pheophytin *a*'	51.4	404, 500, 531, 604, 666	MS, UV	871.5 (100)	593.2 (13)
13	Unknown	58.4				

Figure 3. Typical chromatogram of mulberry leaves (A), digestive organs of silkworm (B), and fresh silkworm excreta (C) elution profile by RP-HPLC of the chlorophyll derivative products. Peak numbers are identified in the accompanying tables. The separation conditions are described in the text.

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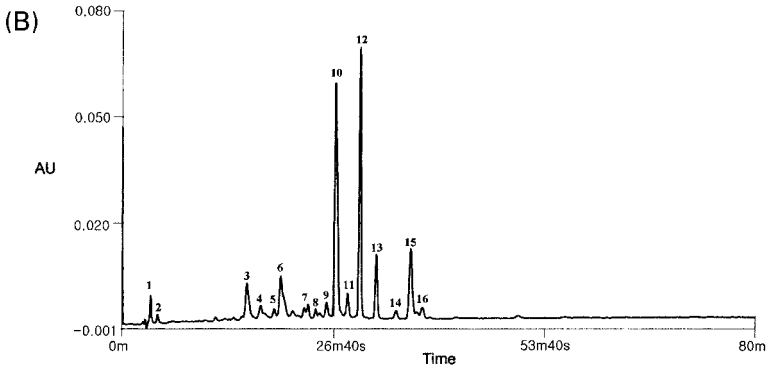


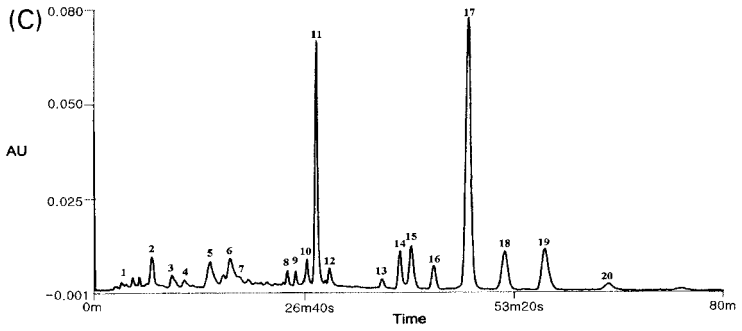
No	Pigment	Time (min)	Absorbance maxima (nm)	Identification	$[M+H]^+$	Main fragment ions
2	Unknown	4.2				
3	Unknown	15.4				
4	Pheophorbide *a* Me	17.2	414, 435, 535, 608, 653	MS, UV	607.1 (100)	
5	Pheophorbide *a'* Me	19.5	414, 435, 535, 608, 653	MS, UV	607.1 (100)	
6	Echinenone	19.5	409, 443, 566, 654	MS, UV	551.4 (100)	
7	Neoxanthin	22.5	414, 435, 464	UV		
8	*cis*-Neoxanthin	23.2	415, 436, 460	UV		
9	Unknown	25.4				
10	Chlorophyll *b*	26.4	458, 595, 648	standard, MS, UV	907.5 (100)	629.1 (35)
11	Chlorophyll *b'*	28.5	458, 595, 648	UV		
12	Chlorophyll *a*	29.5	410, 426, 614, 664	standard, MS, UV	893.5 (100)	615.1 (16)
13	Unknown	32.4				
14	Unknown	34.2				
15	Unknown	36.2				
16	Pheophytin *b*	37.5	416, 441, 468, 578, 655	MS, UV	885.4(100)	607.3(25)

Figure 3. Continued.

(*continued*)

Photosensitizing Effects Against HL-60 Cells of Isolated Pheophorbide-*a* and Pyropheophorbide-*a* from Silkworm Excreta

In phase contrast microscopy, HL-60 cells treated with a photosensitizer fraction (containing both pheophorbide-*a* and pyropheophorbide-*a*) and red light exhibited membrane blebbing and cell shrinkage in more than half of the cells (Fig. 5). These findings coincided with the results observed in MTT assays in which the cell death was scored 1 hr after light irradiation, whereas HL-60 cells treated with other samples survived. The dependence on light of



No	Pigment	Time (min)	Absorbance maxima (nm)	Identification	$[M+H]^+$	Main fragment ions
2	*a*-cryptoxanthin	7.1	422, 432, 479	UV		
3	Pheophorbide *a*	8.5	405, 502, 666	Standard, MS, UV	593.6 (100)	535.8 (40), 517.3 (50)
4	Canthaxanthin	11.2	407, 479, 535	MS, UV	565.1 (100)	
5	Pyropheophorbide *a*	14.0	412, 509, 540, 613, 668	Standard, MS, UV	535.2 (100)	517.3 (10), 488.9 (15)
6	Pyropheophorbide *a* Me	17.9	406, 508, 606, 666	MS, UV	607.1 (100)	
7	Echinenone	19.3	409, 443, 566, 654	MS, UV	551.4 (100)	
8	Unknown	24.2				
9	Neoxanthin	25.2	414, 435, 464	UV		
10	*cis*-Neoxanthin	26.5	415, 436, 460	UV		
11	Chlorophyll *b*'	28.7	458, 595, 648	UV		
12	Unknown	29.4				
13	Pheophytin *b*	36.2	416, 441, 468, 578, 655	MS, UV	885.4(100)	607.3 (25)
14	Hydroxypheophytin *a*	38.4	448, 479, 566, 654	MS, UV	887.4 (100)	869.2 (20), 609.2 (5)
15	Hydroxypheophytin *a*'	40.1	448, 479, 566, 654	MS, UV	887.4 (100)	869.2 (20), 609.2 (5)
16	Pheophytin *a*	43.0	404, 500, 531, 604, 666	MS, UV	871.5 (100)	593.2 (13)
17	Pheophytin *a*'	47.2	404, 500, 531, 604, 666	MS, UV	871.5 (100)	593.2 (13)
18	Unknown	52.5				
19	Unknown	57.1				
20	Unknown	65.1				

*HPLC conditions: pressure, 1000 - 800 PSI; temperature, 20 ; flow rate, 1.0 mL/min; and detection, 430 nm.

Figure 3. Continued.

cytotoxicity by pheophorbide-*a* and pyropheophorbide-*a* was demonstrated by observing the survival of cells protected from light. Pheophorbide-*a* and pyropheophorbide-*a*-PDT induced an apoptosis response in HL-60 cells. As shown in Fig. 5, the control (A), the cells treated with light only (B), and the cells treated with pheophorbide-*a* and pyropheophorbide-*a* fraction only (C) exhibited normal cell morphology. Only in cells treated with pheophorbide-*a* and pyropheophorbide-*a* fraction and light irradiation (D) were apoptotic changes including membrane blebbing, noted in Fig. 5, by confirming laddering of fragmented DNA from the HL-60 cells, the cell death process was assumed to be the apoptosis.

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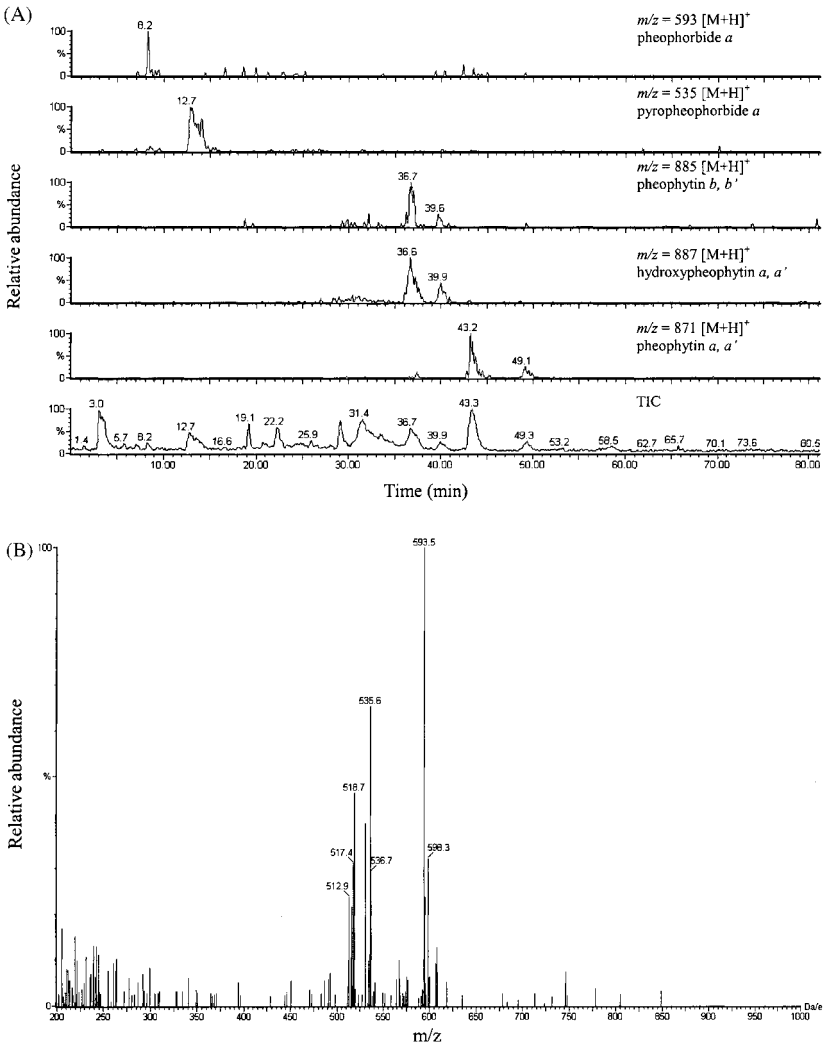

Figure 4. HPLC-APCI-MS analysis of the silkworm excreta using a RP-HPLC column (A), positive ion APCI mass spectrum of pheophorbide-*a* (B), and pyropheophorbide-*a* (C). Mass spectrum of pheophorbide-*a* and pyropheophorbide-*a* extracted at the maximum of the peak eluting at 8.0–8.2 min and 12.7–14.1 min.

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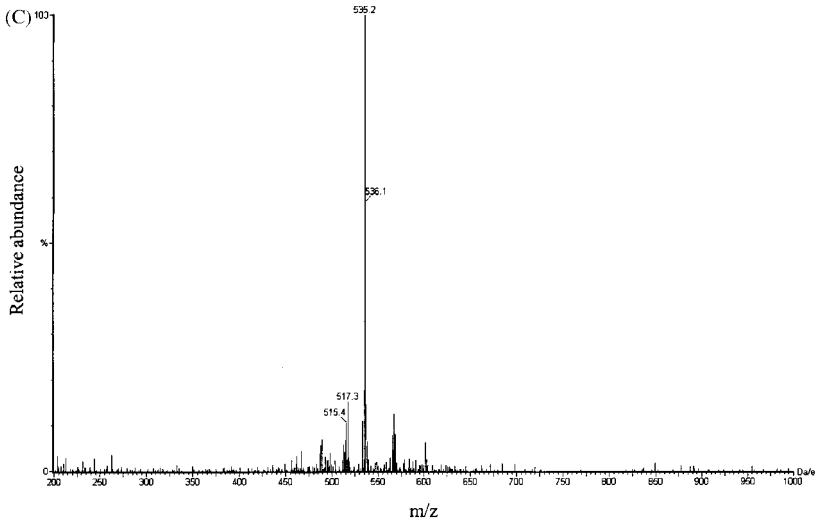

Figure 4. Continued.

CONCLUSION

Photodynamic therapy (PDT) involves administration of a tumor-localizing photosensitizing agent, which may require metabolic synthesis (i.e., a prodrug), followed by activation of the agent by light of a specific wavelength. This therapy results in a sequence of photochemical and photobiological processes that cause irreversible photo damage to tumor tissues. Results from pre-clinical and clinical studies conducted worldwide over a 25-year period have established PDT as a useful treatment approach for some cancers.[23]

In this study, we showed that the components of peaks 3 and 5, (pheophorbide-*a* and pyropheophorbide-*a* from silkworm excreta, respectively, exhibited a notable PDT effect against HL-60 cells (Fig. 5). Thus, pheophorbide and pyropheophorbide compounds may be useful as new photosensitizers in PDT, and silkworm excreta may be a source of crude materials.

We analyzed pigments from mulberry leaves, from the digestive system of silkworms, and from silkworm excreta using a HPLC-UV-APCI-MS. The pigment composition in silkworm excreta has not been previously reported. Pheophytin-*a*, -*b* and hydroxypheophytin-*a*, -*a*′ from silkworm excreta did not show any PDT effect, whereas pheophorbide-*a* and pyropheophorbide-*a* did exhibite a PDT effect against HL60 cells. The structural difference

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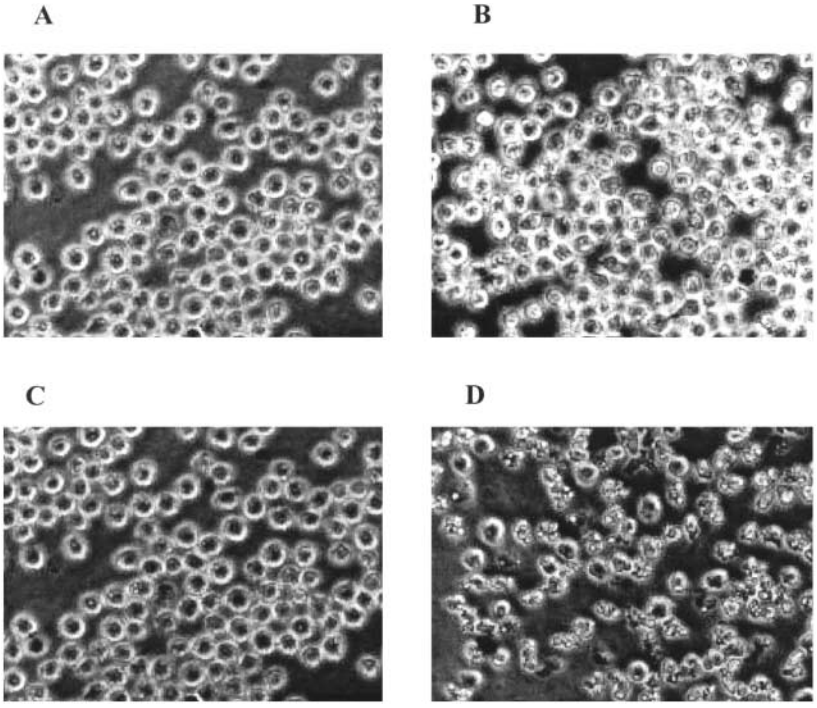

Figure 5. Pheophorbide-*a* and pyropheophorbide-*a*-PDT induced apoptosis response of HL-60 cells: control (A), cells treated with light only (B), cells treated with pheophorbide-*a* and pyropheophorbide-*a* fraction only (C), which exhibited normal cell morphology. Apoptotic changes, including membrane blebbing, were prominent only in cells treated with pheophorbide-*a* and pyropheophorbide-*a*-PDT (D).

accounting for the biological activity for each component was the hydrophilicity of R_2, as shown in Fig. 1. In the pyropheophorbide-*a* analogues, such as methyl esters or carboxylic acids, photosensitizing efficacy increased with length of the alkyl ether side chain. Recently, we has modified R_2 groups of pheophorbide-*a* and pyropheophorbide-*a*, and investigated their PDT efficacy.

The extracts from silkworm excreta aged for more than 1 year in nature showed a stronger PDT efficacy than that from silkworm excreta collected within 24 hrs of analysis. From this, one could infer that stored silkworm excreta may have been fermented by microorganisms, such as fungi, which produced metabolites, such as enzymes, that are capable of further degrading chlorophyll into pheophorbide or pyropheophorbide.

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