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Pheophytin Content and Fibrinolytic Activity of Silkworm Feces in the Different Larval Stages of Silkworms

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In order to find potential anticancer agents, we extracted pheophytin in the silkworm feces from various larval stages by water, chloroform and methanol extraction. The cytotoxicity of the pheophytin extracts of various silkworm feces was measured in the CT-26 cells originated from murine metastatic colon cancer, by dye uptake assay. The cytotoxicity of those pheophytins in 2nd, 3rd and 4th instars was better than remaining larval stages. The *in vitro* anticoagulant and fibrinolytic activities of ethanol extract from varietal mulberry leaves, mulberry branches and silkworm feces and pheophytin extracts from silkworm feces obtained at various larval stages were evaluated in order to find effective therapeutic drugs for the treatment of myocardial and cerebral thrombosis. The fibrinolytic activity was tested using the activated partial thromboplastin time (APTT) and thrombin time (TT) was measured for blood clotting activity. With regards to the fibrinolytic system, ethanol extracts of silkworm feces were better than varietal mulberry leaves and mulberry branches. The pheophytin extracts from 7th days of 5th instar contained the highest percentage of pheophytin and good fibrinolytic activity.

Key word: Pheophytin, Silkworm feces, Fibrinolytic activity

Introduction

Sunlight-dried feces of silkworm (*Bombyx mori*) obtained from 3rd - 4th instar larvae have been used in China as the remedy for palsy, blood circulation, removal of fever, healthy eyes, headache, itching, arthritis and etc. (Cui *et al.*, 2002).

In a previous report, ethanol extracts from freeze-dried, the 3rd day of 5th instar feces showed more potent anticancer activity than those of other mulberry leaves, mulberry branches and other 5th silkworm instar larvae feces (Ahn *et al.*, 2001). It contains phytol (0.25~0.29%), β-sitosterol (1.5%), cholesterol, ergosterol, tetracosanol, lupeol, bombyrenone, β-sitosterol-β-glucoside, carotene, heteroauxin, Vitamin A, B, E, chlorophyll, amino acid and etc.

Recently, Lee *et al.* (1990) reported that certain chlorophyll derivative (CpD) frcations from TLC of silkworm metabolites were effective for photodynamic therapy (CpD) in test animals, exhibiting killing of ascites tumors. The essential CpD component was determined to be mainly 10-hydroxypheophytin a, little b, chlorophyllide and pheophorbide including the a~d forms of chlorophyll by reversed-phase high-performance liquid chromatography with photodiode array and fluorescence detection. Several authors have also suggested the exsitence of a Mg-releasing enzyme (Mg-dechelatase) which would catalyze the removal of Mg^{2+} ion from the tetrapyrrolic ring (Almea *et al.*, 2000).

In this study, we report upon cytotoxicity on cancer cell, pheophytin content and fibrinolytic activity of silkworm feces obtained in different developmental stages of silkworm larvae.

Materials and Methods

Preparation of Test Solution

Each (2nd~5th instar larvae) silkworm feces was freezing-dried. The dried sample (50 g) was extracted with 625 ml of distilled water by incubation at 50°C for 2.5 hrs with

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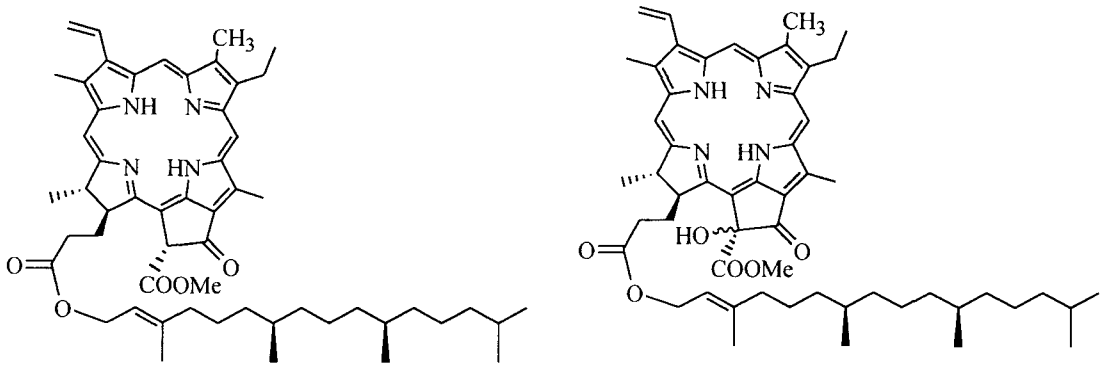

Fig. 1. Structure of pheophytin a and 10-hydroxypheopytin a.

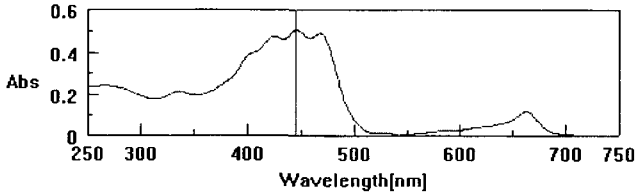

Fig. 2. Profile of UV spectrum.

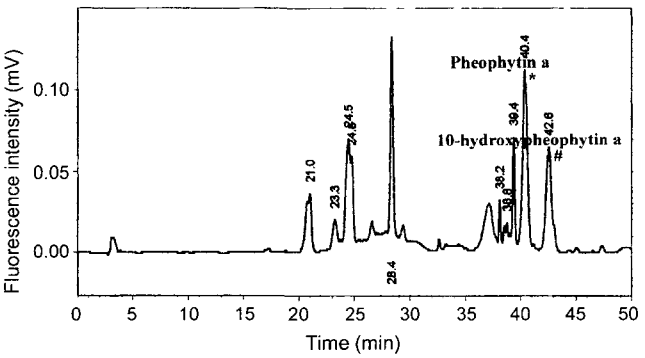

Fig. 3. HPLC chromatography of the pheophytin from 3rd day of 5 instar by fluoresence detector.

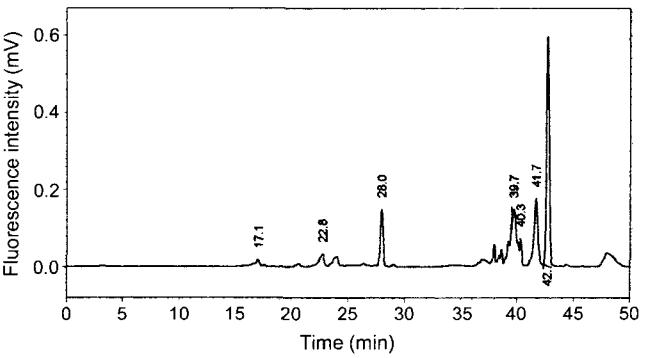

Fig. 4. HPLC chromatography of the pheophytin from 7rd day of 5 instar by fluoresence detector.

mechanical stirring. The extract was filtered through four layers of gauze and the filtrate was then centrifuged for 10 min at 7000 g in a Suprazik centrifuge (Hanil, Incheon, Korea). The pellet, which contained most of the chloroplast material, was suspended in 435 ml of methanol and 188 ml of $CHCl_3$ and incubated at 50°C for 1.5 hrs with mechanical stirring. In an aliquot of these extracts was investigated their spectra maxima (UV max). These extracts were concentrated by evaporator (Buchi Co., Ltd.) and resuspended in chloroform and methanol (1 : 9) for HPLC (Spectra system FL3000). The pheophytin (pheophytin a and 10-hydroxypheophytin a) obtained from Department of Kangwon University, was used as retention time standard. All procedures were performed under shading light.

Chromatography

The HPLC equipment consisted of a Thermo Spectra Products (San Jose, USA) liquid chromatographic system equipped with P4000 operated from a Spectrasytem controller. Spectrasystem fluorescence FL3000 and a Spectrasystem UV 3000 photodiode UV-Vis detector were used on line. The fluorescence detector was operated at 430 nm (excitation) and 670 nm (emission). Spectral data from PAD system were recorded between 430 and 670 nm. Analytic separations were performed on a 5 μm Luna C^{18} column (25 cm × 0.4 cm, I. D.) (Phenomex, USA). Semipreparative separations were carried out on Luna C^{18} column. Silkworm feces pigments were eluted using a linear gradient in 30 min from 100% solvent A, (80% methanol in 1 M ammonium acetate) to 100% solvent B (80% methanol in acetone). Solvent B was maintained until the pigments were completely eluted. The flow-rate was 0.8 ml/min with the analytic column. Isolated pigments were identified according to the retention time of standard references (Braumann and Grimme, 1981).

Effects on Coagulation Systems

Human plasma obtained from the Blood Bank of Seoul National University Hospital was used for measuring clotting time in both activated partial thromboplastin time (aPTT) and thrombin time (TT). The clotting time tests were performed on a Beckton Dickenson BBL Fibrosystem (Cockeysville, U. S. A.). In brief, a mixture containing 40 μl of test solution and 80 μl of prewarmed plasma for one minute was incubated for three minutes under stirring, then dropped into 0.02 M calcium chloride solution at 37°C. When the clot was formed, aPTT was measured. For the measurement of TT, an equal volume of thrombin (10 U/ml) and test insect fractions were mixed and incubated at 37°C for 5 minutes.

Measurement of Cytotoxicity

The cytotoxicities of the extract solutions of silkworm feces were tested against CT-26 colon cancer cell line using XTT {sodium 3'-[1-(phenylamino-carbonyl)-3, 4-tetrazolium]-bis (4 methoxy 6-nitro) benzene sulfonic acid hydrate} kit solution (Boehringer Mannheim), as described previously (Geldof *et al.*, 1999). All measurements were performed in triplicate. The IC_{50} (50% inhibitory concentration; mg/ml) was defined as the fraction of extract that caused a 50% inhibition of cell viability compared with the control.

Results and Discussion

Pheophytin content of silkworm feces according to silkworm larvae stage

Chlorophyll derivatives include a blood spectrum of green-grey-brown pigments of very different polarities. Fig. 1 shows the structures and names of the different compounds considered. Pheophytins and chlorophyllides are Mg-free (replaced by 2H) and phytyl-free derivatives, respectively of the parent chlorophylls, whereas the pheophorbides are free of both Mg and phytol.

As the results, the 2nd instar silkworm larvae feces contained relatively more pheophytin content per mg than silkworm feces obtained at other larvae stages, but 7th day of 5th instar silkworm larvae feces had more content than others when calculated by total silkworm feces amount. The result indicated that the more mulberry leaf silkworm ingested the more chlorophyll (pheophytin) in the silkworms (Table 1).

Table 1. UV absorption at 665/434 nm of the pheophytin extract from silkworm feces in various instar ages under UV at maximal wavelength

Instar age/day	434 nm	665 nm
2/0	1.91	1.09
3/0	0.45	0.07
4/2	1.13	0.43
4/3	1.80	0.62
5/2	0.86	0.27
5/3	0.47	0.12
5/4	0.45	0.09
5/6	1.58	0.42
5/7	3.18	1.30

Table 2. HPLC analysis of pheophytin a and 10-hydroxypheophytin a of the extract of silkworm feces, silkworm and mulberry leaves

Instar age/day	Pheophytin extract (g/50g crude silkworm feces)	Pheophytin extract 1 mg		Pheophytin a and 10-Hydroxypheophytin a (mg/50 g crude silkworm feces)
		Pheophytin a (μg)	10-Hydroxypheophytin a (μg)	
Pheophytin standard (5 mg/ml)		140.58	127.42	
Silkworm feces				
2/0	1.863	4.23	0	7.80
3/0	0.990	200.98	201.47	398.43
4/2	1.253	91.66	447.08	675.04
4/3	0.730	307.23	115.75	308.78
5/2	0.768	71.97	161.54	130.92
5/3	0.688	180.93	222.31	277.43
5/4	0.594	68.58	267.32	199.52
5/6	1.182	205.09	340.72	645.15
5/7	1.824	471.62	794.80	2309.95
Silkworm	0.187	792.58	747.85	288.06
Mulberry leaves	0.754	0.51	173.74	131.38

Table 3. Measurement of cytotoxicity of ethanol extract (a) and pheophytin (b) of silkworm feces to CT-26 cell lines

(a)

Instar age/day	IC_{50} (mg/ml)
1/0	100
2/0	100
3/0	45.94
4/2	21.98
4/3	100>
5/1	19.68
5/2	15.09
5/3	11.78
5/5	21.81

(b)

Instar age/day	2/0	3/0	4/2	4/3	5/2
IC_{50} (mg/ml)	0.08	0.89	0.34	>100	>100
Instar age/day	5/3	5/4	5/6	5/7	MMC
IC_{50} (mg/ml)	>100	>100	>100	321.1	0.64

Anticancer activity of ethanol and pheophytin extract of silkworm feces according to silkworm larval stages

The cytotoxic activities of ethanol and pheophytin extract of silkworm feces were evaluated by XTT assay (Table 3a and 3b). The IC_{50} values of the pheophytin of silkworm feces according to silkworm larval stages, were 0.008 mg/ml (2nd instar), 0.89 mg/ml (2nd day of 4th instar) and 0.89 mg/ml (3rd instar) compared mitomycin C (0.64 mg/ml) for CT-26 colon cancer cells.

Fibrinolytic activity of pheophtin from silkworm feces according to silkworm laeval stages.

The majority of three ethanol extracts (varietal mulberry leaves and mulberry branches from 16 varietal mulberries/various 2nd ~ 5th larval stages and silkworm feces) displayed markedly increased anticoagulant activity as shown in Table 4, 5, 6. In present study, the *in vitro* anticoagulant activities of ethanol extract from varietal mulberry leaves, mulberry branches and silkworm feces and pheophytin extracts from silkworm feces from various larval stages were evaluated by activated partial thrombine time test and thrombin time test. Thrombin time test, however, did not show anticoagulant activity in the varietal samples, but APTT test showed that ethanol extract of mulberry leaves and branches of Chengwoonppong revealed positive activity. Of these the pheophytin extract of silkworm feces according to silkworm larval stages, 7th day of 5th instar showed good activity on the fibrinolysis system (0.1 mg/ml concentration) (Table 7). The ethanol extracts of the silkworm feces according to silkworm larval stages exhibited better fibrinolytic activity than those of mulberry leaves. Further, 3rd instar silkworm feces had better fibrinolytic activity than other

Table 4. APTT in the ethanol extracts of the varietal mulberry leaves and branches (sec.)

Sample number and variety	Mulberry leaves	Mulberry branches
Control (saline)	68.0	
1. Keryangppong	112.5	124.0
2. Cheongilppong	162.0	97.5
3. Shinilppong	112.0	88.9
4. Susungppong	147.0	105.5
5. Sugyeppong	107.0	104.0
6. Shinkwangppong	145.0	69.5
7. Cheongwoonppong	156.5	124.5
8. Milsungppong	136.3	154.5
9. Sangilppong	139.5	160.5
10. Daeryukppong	115.0	146.5
11. Suwonppong	132.5	115.5
12. Yongchunppong	129.0	118.0
13. Gumsulppong	140.0	131.5
14. Subongppong	139.0	147.0
15. Cheogolppong	148.5	124.5
16. Hongolppong	129.7	127.0

*Test materials: 1 mg/ml in 0.4% DMSO saline solution.
*APTT: activated partial thromboplastin time (sec.).

Table 5. APTT of the ethanol extracted silkworm feces

Drying method	5th instar age	APTT (sec.)
Control		68.0
Shade dry	2nd day	142.0
	3rd day	129.5
	4th day	145.0
Freezing dry	3rd day	151.0
	matured larvae	186.0

*Test materials: 1 mg/ml in 0.4% DMSO saline solution.

Table 6. APTT test of pheophytin extract of silkworm feces

Instar age/day	APTT (sec.)	Instar age/day	APTT (sec.)
2/0	89.3	5/4	76.0
3/0	119.0	5/6	91.0
4/2	108.0	5/7	105.0
4/3	98.0	Silkworm	87.8
5/2	102.0	Mulberry leaves	41.5
5/3	78.4		

*Test materials: 0.1 mg/ml in 0.4% DMSO saline solution.

stages as shown in Encyclopedia of Chinese drugs (Jungyakdaesajun, 1977).

In conclusion, according to our experiment, silkworm feces are as potent as natural crude drugs in their effects on anticancer and fibrinolytic activity. The present results justify further studies to identify mechanism of its action.

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