Indian Journal of Biochemistry & Biophysics
Vol. 51, October 2014, pp. 388-395

Organ-specific distribution of chlorophyll-related compounds from dietary spinach in rabbits

Ching-Yun Hsu[1], Tsan-Huei Yeh[2], Meng-Yuan Huang[2], Shene-Pin Hu[3], Pi-Yu Chao[4*] and Chi-Ming Yang[2*]

[1]Department of Nutrition and Health Sciences and Research Center for Industry of Human Ecology, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan

[2]Biodiversity Research Center, Academia Sinica, Nankang, Taipei 115, Taiwan

[3]School of Nutrition and Health Science, Taipei Medical University, Daan, Taipei 110, Taiwan

[4]Department of Health and Nutrition Sciences, Chinese Culture University, Hsulin, Taipei 111, Taiwan

Received 27 November 2013; revised 19 August 2014

The distribution of chlorophyll-related compounds (CRCs) derived from dietary spinach was investigated in different organs the rabbits. The rabbits in the experimental group consumed 100 g of freeze-dried spinach powder after a 24 h fasting period and sacrificed 2, 4, 8, 12 and 24 h later and in the control group sacrificed after the 24 h fasting period. The main CRCs in the liver were found to be chlorophyll (Chl a) and b, chlorophyllide (Chlide) a and b, pheophytin (Phe) a and b and pheophorbide (Pho) a and b, which reached their peak values at 8 h post-feeding. The gallbladder contained mainly Chlide a and a', Pho a and a', Pho b and b', which peaked their values at 2 h post-feeding. Pho a and b were consistently observed in the blood and peaked at 12 h post-feeding. The earlier appearance of Chlide a', Pho a' and Pho b' in the gallbladder compared to the liver indicated that these CRCs were compartmentalized differently and might undergo the same type of vectorialized transport as characterized for the bile salts. Pho levels peaked later in the blood compared to the liver, suggesting that Pho might be released into the peripheral blood circulation from the liver. In conclusion, Chlide and Pho were the principal Chl metabolites in the rabbits. Our data may expand our understanding of the metabolism and biodistribution of CRCs in the human body. A number of biological functions, including anti-oxidation, anti-tumor and anti-aging have recently been attributed to CRCs, it will be interesting to explore, if the binding of Chlide and Pho to other nutrients or trace metal ions in the body mediate their biological functions.

Keywords: Chlorophyll-related compounds, Catabolites of chlorophyll, Liver, Gallbladder, Distribution

Chlorophyll (Chl) is a tetrapyrrole compound containing a central Mg^{2+} ion and an isoprenoid phytyl side chain. It can be degraded to form a number of compounds including i) pheophytin (Phe), a Chl derivative lacking Mg^{2+}; ii) chlorophyllide (Chlide), a derivative lacking phytyl group and is formed by chlorophyllase-mediated hydrolysis, and iii) pheophorbide (Pho), a Chl derivative lacking both Mg^{2+} and phytyl groups[1]. Names of Chl derivatives containing a prime symbol, such as Pho a' and Pho b', refer to epimerization at carbon-13, which can occur in living plants, as well as after extraction from the plant[2]. All these photosynthesis-related pigments are named as chlorophyll-related compounds (CRCs).

There are a number of compelling reasons to identify CRCs present in plants and animals, as well as in humans during digestion and metabolism. First, the Chl degradation pathways in plants, as well as the dietary Chl metabolism in humans remain poorly characterized[3]. Second, the specific derivatives of plant Chl and bacterial Chl, such as palladium-bacteriopheophorbide (WST-09)[4-7] and 2-[1-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH)[8,9] have shown efficacy as photosensitizing agents during anti-tumor therapy. Third, it has been suggested that anti-tumor activity of certain Chl derivatives is mediated via inhibition of absorption of specific carcinogens[10,11].

The biodistribution and catabolism of CRCs have been assessed in animals and humans. In a study on the biodistribution of palladium-bacteriochlorophyll derivative WST11 the hepatic clearance has been demonstrated as the major pathway in mice with melanoma[12]. Similarly, the palladium-bacteriopheophorbide derivative WST09 has been

*Corresponding authors:
E-mail: cmyang@gate.sinica.edu.tw (Chi-Ming Yang)
E-mail: pychao@faculty.pccu.edu.tw (Pi-Yu Chao)

Abbreviations: Chl, chlorophyll; CRCs, chlorophyll-related compounds; Chlide, chlorophyllide; Phe, pheophytin; Pho, pheophorbide; HPPH, hexyloxyethyl]-2-devinylpyropheophorbide-a; 2-[1-hexyloxyethyl]-2-devinylpyropheophorbide-a (HPPH).

shown to accumulate in the plasma, kidney and liver and is rapidly cleared from each of these tissues in EMT6 tumor-bearing mice and no significant accumulation has been observed in skin, muscle or tumor tissues. Using rats, Bellnier *et al.*[8] have provided detailed time-course data of administered photodynamic sensitizer 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) and have demonstrated its presence in the plasma, skin, liver, spleen, kidney, feces and urine. Moreover, HPPH does not produce marked skin sensitivity and is efficacious in mediating photo-treatment against subcutaneous tumors.

CRCs distribution in humans is not completely understood. Dandler *et al.*[13] characterized the distribution of Chl- and bacterio-chl-derived photosensitizers in human plasma and found low levels of cell-bound CRCs; additionally, low-density lipoproteins (LDL), high-density lipoproteins (HDL) and high-density proteins (HDP) exhibit different levels of binding to CRCs. However, these results have been questionable because the blood has not been freshly isolated and obtained from a pool of anonymous donors. Another study has shown nearly 100% binding of 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a to plasma proteins in the cancer patients[14].

Uptake of CRCs from spinach homogenates has been investigated in Caco-2 cells[15,16]. It is reported that Chl pigments are transferred from the ingested food to micelles, with Pho a being the most micellarized derivative[16]. In addition, elevated levels of allomerized Phe a and Phe b are found after exposure of spinach homogenates to simulated gastric digestion[15]. Caco-2 cells have also been used to study uptake of CRCs from the peas[17]. Deesterification of alcohol phytol group is shown to result in increased transfer to the aqueous micellar fraction and uptake by Caco-2 cells[16].

Previous studies have mainly focused on the distribution of individual CRCs after intravenous administration. In the present study, we have investigated the distribution of CRCs derived from dietary spinach in the rabbits. Our data may expand our understanding of the metabolism and biodistribution of CRCs in the human body.

Materials and Methods

Experimental animals and diet

Two-months-old weaned female New Zealand white rabbits (n = 15), weighing 1.8-2.2 kg were purchased from the Experimental Animal Center of the Affiliated Hospital of the National Taiwan University. The rabbits were acclimated for a week after arrival. During acclimatization, rabbits received rabbit feed (120 g/day) and had free access to drinking water and were exposed to 12 h of light and 12 h of dark. The body weights were recorded daily. After the acclimatization period, rabbits were randomly divided into the control group (n = 2) and the experiment group (n = 13). Animals in the control group were sacrificed after a 24-h fast. Animals in the experimental group were fed 100 g of freeze-dried spinach powder after 24 h of fasting and sacrificed at 2 (n = 2), 4 (n = 3), 8 (n = 3), 12 (n = 3) and 24 h (n =2). The freeze-dried spinach powder was prepared from fresh spinach which was freeze-dried, ground into powder and stored at -70°C.

Preparation of CRCs standards

Chl a and b were purified by chromatography using a CM-Sepharose CL-6B® as previously described[18]. Phe was obtained by adding several drops of acetic acid into Chl a and b. Chlide a and b, Pho a and b were prepared by adding chlorophyllase-containing extraction fluid into Chl a, Chl and Phe, respectively. Purified Chlide and Pho were obtained after 30 min of reaction. Purified Chl a and b were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as a standard control for HPLC.

Pheophytinization

Chl a and b were dissolved in ether after which a few drops of acetic acid were added. The solution was dried by a stream of nitrogen gas for approx. 2 min and then stored at -70°C until later use.

Chlorophyllide and pheophorbide

Reaction reagent (9 ml) was added into the aforementioned enzyme-containing powder and the sample was mixed well. The supernatant was collected and 10 mg Chl a was added. After sonication, the sample was filtered using filtration and acetone was used to rinse the residue left on the filter paper. The filtrate was collected and NaCl was added to saturate the filtrate. Ether was added for extraction and the extract was dried with nitrogen gas and re-dissolved in acetone for the CM-Sepharose® column chromatography. The analysis consisted of three steps: i) Unreacted substances were eluted using acetone at the beginning, ii) 5% methanol in acetone was used as wash buffer for pheophorbide, and iii) 25% methanol in acetone was used as wash buffer for chlorophyllide (the yield rate was about 70~90%).

Preparation of chlorophyllase and activity analysis

Fresh milky spot ficus leaves were quickly frozen in liquid nitrogen and ground; 350 ml of -20°C acetone was added for the extraction. The extracts were filtered and dried with nitrogen gas and stored at -20°C. A sample of 160 mg dry powder was collected, and 5 mL extraction reagent was added. A polytron homogenizer was used to homogenize the sample and the sample was cultured at 37°C for 2 h, followed by centrifugation at 15,000 *g* for 10 min. The enzyme-containing supernatant was collected. A sample of 0.5 mL supernatant was collected and 2 mL of the reaction reagent and 0.2 mL of ether (0.6 μmol Chl) were added and mixed well at 37°C. Acetone/*n*-hexane/10 mM aqueous KOH (4:6:1; v/v) was added in the mixture and the sample was shaken well. The sample was separated into two layers; the upper layer contained hydrophilic Chlide in the acetone phase and the lower layer contained hydrophobic Pho in the hexane phase. The two layers were analyzed using a spectrophotometer.

Animal sample preparation (*Blood, urine and feces*)

Fecal samples were collected before the rabbits were anesthetized. Since the rabbits did not have any bowel movement at the 2 and 4 h time points, sample data were only obtained at 8, 12 and 24 h post-feeding. Blood samples were collected after the rabbits were anesthetized. A needle was inserted into the heart and blood was collected into a tube containing EDTA. The samples were pre-chilled at 4°C and centrifuged at 3,000 rpm at 4°C for 15 min. The supernatant was equilibrated using D = 1.006 NaCl and centrifuged at 44,000 rpm at 4°C for 16 h. The upper white thin layer contained very low density lipoproteins (VLDLs). The sample was equilibrated with D = 1.063 NaBr and centrifuged at 44,000 rpm at 4°C for 22 h. The upper white thin layer contained LDL. Urine samples were collected directly from the bladder using a needle.

Organs

Rabbits were anesthetized using ether and sacrificed by cervical dislocation. Liver, kidney, pancreas and gallbladder were collected and rinsed with phosphate-buffered saline (PBS) in order to prevent the influence of heme in the blood. A needle was inserted into the portal vein and PBS was injected; the liver was perfused until it was white. The prepared organs were weighed and rapidly frozen in liquid nitrogen. The freeze-dried organs were ground into powder in liquid nitrogen and stored at -70°C.

Extraction of CRCs in animal samples

Freeze-dried samples were collected and 80% acetone was added in a ratio of 1:20 (w/v) for grinding. Filter paper (TOYO Advantec®; 150 mm) was used to filter impurities and then a crude extract of CRCs was obtained. The crude extract was placed in a separating funnel and ethyl ether (1:20 v/v) and 10% KCl (1:10, v/v) were added. The sample was mixed and then left to stand for a few minutes until it separated. The upper layer fluid was collected. Deionized water (1:20, v/v) and 10% KCl (1:10, v/v) were added and mixed and sample was then allowed to rest for several minutes. After separation, the lower layer was removed and the upper layer was collected. This experimental procedure was repeated once. The upper layer extracts were mixed and ethyl ether was removed with rotary evaporation (Buchi Rotavapor R-124, Firsttec Scientific B401L). The dried sample was then dissolved into ether to make 1 mL of pure pigment solution. The solution was filtered with Anylon filter membrane (0.45 μm aperture; Nylon acrodisk, Gelman) and a sample of 25 μL of the filtrate was collected for high-performance liquid chromatography (HPLC) at A_{660nm}.

HPLC and spectroscopy

Analytical separations were performed on a 5 μm Spherisorb® ODS-2 250 * 40 mm column (C18) using the Waters Model 510 (Millipore-Waters, Milford, MA) high pressure pump. The flow rate was controlled with a Waters Model 680 automated gradient controller. Waters U6K injector and Waters Dual λ Absorbance Detector (Model 2487) were used. The solvent A was 1 M ammonium acetate/methanol (2/8, v/v) and the solvent B was acetone/methanol (2/8, v/v). Pigments were identified at 660 nm using retention time and visible absorption characteristics. The SISC HPLC analysis system developed by Yehua Technical Institution (Taipei, Taiwan) was used. The absorbance of the pigment solution was measured using Hitachi U-2000 UV-visible spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at 350~750 nm.

Spectral characteristics of CRCs

Chl a and b were purified from the fresh spinach. The other six derivatives, including Chlide a and b, Phe a and b and Pho a and b were obtained using chemical and biochemical methods. Each CRC had a major absorption peak in the blue and red light region. Chl a and its derivatives had significantly different

absorption peaks from those of Chl b and its derivatives. The absorption peaks of Chl a and its derivatives ranged between 660 and 665 nm, while those of Chl b and its derivatives ranged between 640 and 645 nm (Table 1). Thus, 660 nm was used to record the changes of CRCs during component analysis. CRCs with high polarity, including Chlide a and b and Pho a and b were eluted using solvent A at an earlier time. CRCs with less polarity, including Chl a and b and Phe a and b had a longer retention time in the column (Table 2). Several kinds of epimers and allomers were also found in small quantities.

Statistical analysis

Data were expressed as the mean ± standard deviation and statistical significance was analyzed using a one-way analysis of variance (ANOVA), followed by least significant difference (LSD) at the 0.05 significance level. Statistical analysis was two-tailed and conducted using JMP software, version 5.01 (SAS Institute; Cary, NC). The means of three biological replicates are reported.

Table 1—Spectral characteristics of chlorophyll-related compounds[22]

Pigments	Solvent	λ_{max} (nm)	Reference (nm)
Chlorophyll a (Chl a)	Diethyl ether	423 658	430 661
			430 662
	Acetone	422 660	428 663
Chlorophyll b (Chl b)	Diethyl ether	418 447 644	430 452 642
			452 642
	Acetone	420 452 649	453 645
Chlorophyllide a (Chlide a)	Diethyl ether	430 662	428 662
	Acetone		
Chlorophyllide b (Chlide b)	Diethyl ether	595 644	458 594 643
	Acetone		
Pheophytin a (Phe a)	Diethyl ether	503 666	410 505 667
			408 667
	Acetone	665	409 664
Pheophytin b (Phe b)	Diethyl ether	437 521 654	526 598 654
			525 599 654
	Acetone	437 653	430 652
Pheophorbide a (Pho a)	Diethyl ether	406 667	408 505 667
			408 665
	Acetone	406 502 666	
Pheophorbide b (Pho b)	Diethyl ether	430 661	430 525 654
			430 523 656
	Acetone	431 659	

Results

CRCs in spinach

Fresh spinach yielded a total of 1.36 g of Chl a and 0.63 g of Chl b/100 g spinach (wet weight), respectively. The Chl a/b ratio was approx. 2.5 (Fig. 1), which was similar to that reported for the freeze-dried spinach[19]. The major CRCs were Chl a and b, both in fresh as well as freeze-dried spinach.

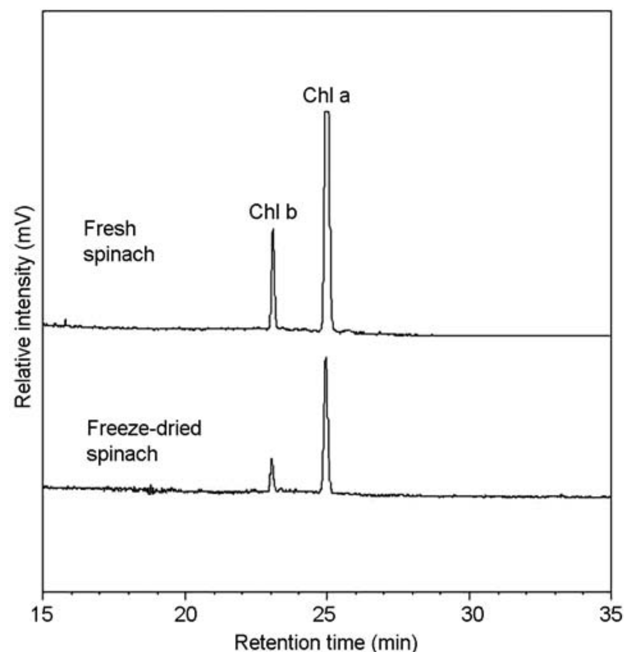

Fig. 1—Comparison of Chl components in fresh and freeze-dried spinach

Table 2—Retention time of chlorophyll-related compounds in HPLC

Pigments	Abbreviation	Retention time (min)
Chlorophyllide b	Chlide b	10.687
Chlorophyllide a	Chlide a	13.903
Pheophorbide b	Pho b	15.923
Pheophorbide a	Pho a	17.487
Pheophorbide a´	Pho a´	17.830
Chlorophyll b allomer	Chl b´	23.383
Chlorophyll b	Chl b	23.663
Chlorophyll b epimer	Chl b´	24.231
Chlorophyll a allomer	Chl a´	24.668
Chlorophyll a	Chl a	25.387
Chlorophyll a epimer	Chl a´	26.153
Pheophytin b	Phe b	29.613
Pheophytin b´	Phe b´	30.550
Pheophytin a	Phe a	33.677
Pheophytin a´	Phe a´	37.998

Other derivatives were not observed in the extract, which was consistent with reports that Chl breakdown products occur only in traces in plants[3].

CRCs in blood

Blood CRCs were mainly Pho a, Pho a' and Pho b (Fig. 2). There was a gradual increase in the concentration of Pho a and b after ingestion of spinach, peaking at 12 h post-feeding and gradually decreasing thereafter. Pho a' was detected at 4 h post-feeding and 2.0 ng/mL concentrations were observed from 12 to 24 h post-feeding.

CRCs in liver

Chl a, Chlide a and b, Phe a and b and Pho a and b were detected in the liver (Fig. 3). There was a gradual increase in the concentrations of Chl a, Chlide a, Pho a, Phe b and Phe b', which peaked at 8 h post-feeding. Dramatic peak concentrations of Chlide a, Pho a, Phe b and Phe b' were found in the liver at 8 h; Phe b and Phe b' were only found in the liver at 2, 4 and 8 h, but were not detected at 12 and 24 h post-feeding. Relatively high Chl a and Phe a baseline (0 h) concentrations were also observed; however, they were not detected in the gallbladder at any time.

CRCs in gallbladder

The gallbladder contained high levels of Chlide a and a', Pho a and a', Pho b and b', which peaked at 2 h post-feeding and then declined (Fig. 4). Surprisingly, the peak concentrations of CRCs in the gallbladder were observed at an earlier time compared to the liver.

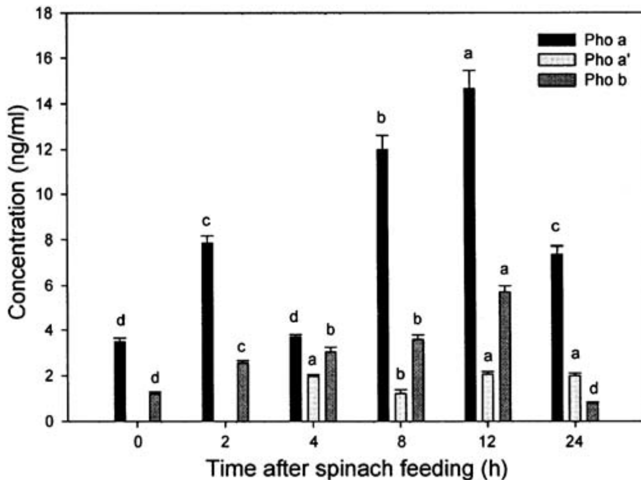

Fig. 2—Concentration of Chl-related compounds from dietary spinach in blood (n = 3/group) [Data were expressed as mean ± standard deviation and tested by ANOVA, followed by LSD test for post-hoc multiple comparisons. Various letters indicate statistical significance between groups. ns, non-significant at P < 0.05]

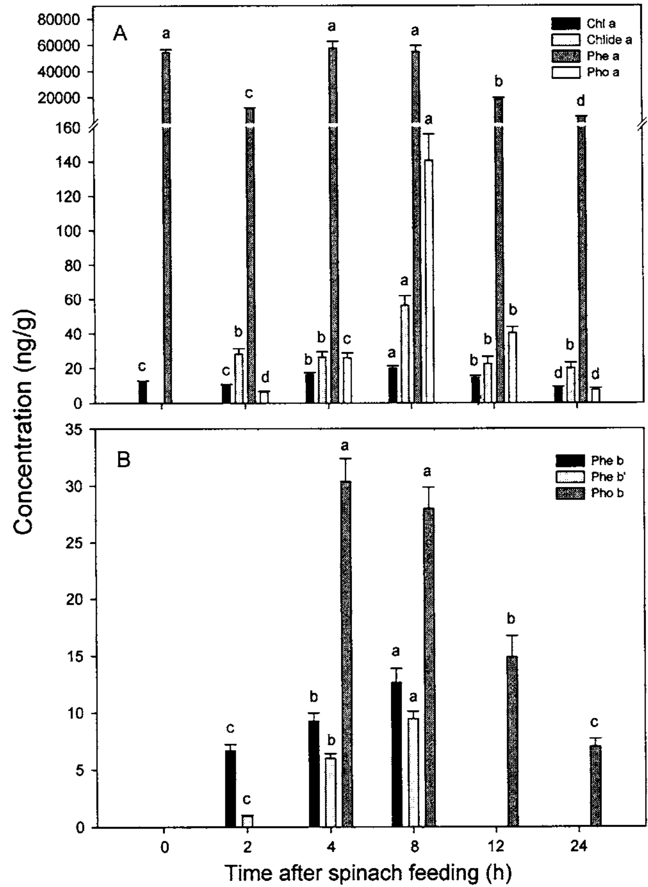

Fig. 3—Concentration of Chl-related compounds from dietary spinach in liver (n = 3/group) [Data were expressed as mean ± standard deviation and tested by ANOVA, followed by LSD test for post-hoc multiple comparisons. Various letters indicate statistical significance between groups. ns, non-significant at P < 0.05]

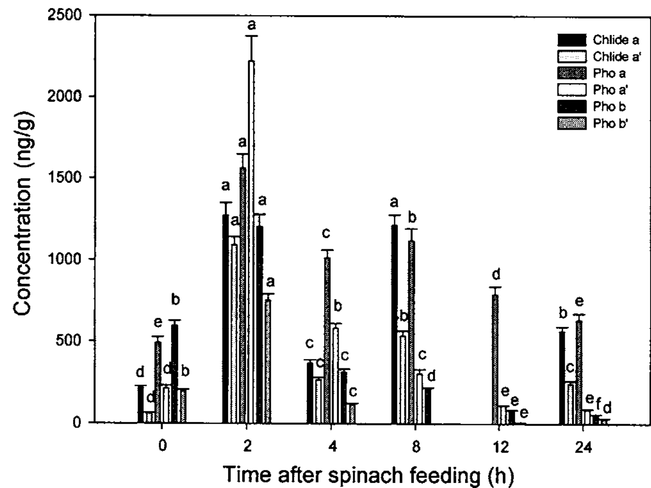

Fig. 4—Concentration of Chl-related compounds from dietary spinach in gall bladder (n = 3/group) [Data were expressed as mean ± standard deviation and tested by ANOVA, followed by LSD test for post-hoc multiple comparisons. Various letters indicate statistical significance between groups. ns, non-significant at P < 0.05]

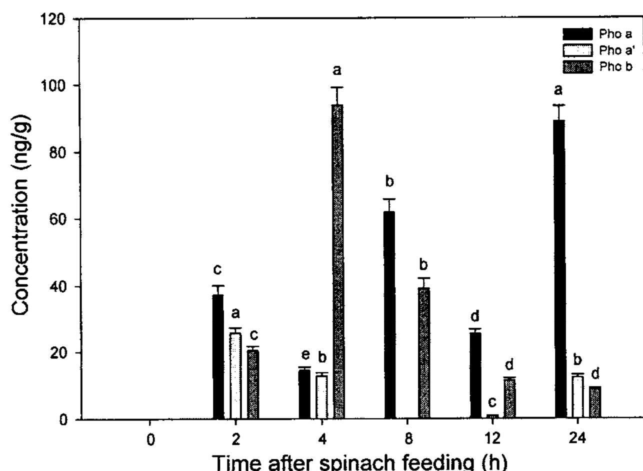

Fig. 5—Concentration of Chl-related compounds from dietary spinach in kidney (n = 3/group) [Data expressed as mean ± standard deviation and tested by ANOVA, followed by LSD test for post-hoc multiple comparisons. Various letters indicate statistical significance between groups. ns, non-significant at P < 0.05]

CRCs in kidney and pancreas

The major CRCs in the kidney were Pho a, Pho a' and Pho b, which were similar to those observed in blood (Fig. 5). Pho b reached peak values at 4 h post-feeding. The concentration of Pho a fluctuated and reached peak values at 24 h post-feeding; Pho a' had peak values at 2 h post-feeding and was almost undetectable at 8 and 12 h, but reappeared at 24 h post-feeding. No CRCs were detected in the pancreatic tissue.

CRCs in urine and feces

The major CRCs detected in the feces were Chl a, Chl a', Phe a, Phe a', Chl b, Chl b', Phe b, Phe b' and Phe b" and Phe'" (Fig. 6). The concentrations of Chl a, Chl a', and Chl b continued to increase and reached their highest levels at 24 h post-feeding. Chl b' reached a peak value at 8 h post-feeding. The concentrations of Phe a and Phe a' were relatively steady throughout the study period. The concentrations of Phe b and Phe b' reached peak values at 12 h post-feeding. CRCs were only found in the urine samples of some rabbits.

Discussion

In this study, we showed two forms of Chl, Chl a and Chl b in fresh and freeze-dried spinach at a ratio of 1.36 g: 0.63 g. We did not detect other Chl derivatives in fresh or freeze-dried spinach, possibly due to their low levels. Our data were consistent with the results of an earlier study[20]. Previous data have shown that Chl a and Chl b are converted to Phe a and

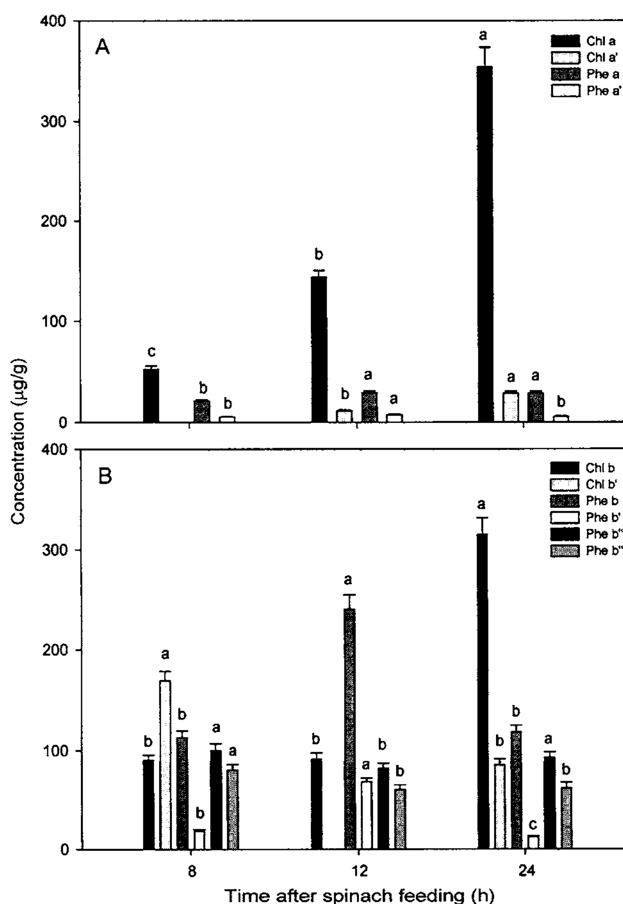

Fig. 6—Concentrations of Chl-related compounds from dietary spinach in feces (n = 3/group) [Data expressed as mean ± standard deviation and tested by ANOVA, followed by LSD test for post-hoc multiple comparisons. Various letters indicate statistical significance between groups. ns, non-significant at P < 0.05]

Phe b, respectively during the digestive process[15]. Although Chl b is thought to be more stable to digestion than Chl a[17], Chl a and Chl b are less stable to the digestive process than Mg-free Chl derivatives[16].

In the present study, we showed the presence of CRCs in the liver and gallbladder, but not in the pancreas of rabbits. Our data differed from a previous study which has shown the presence of Pho a in the gut, lung and pancreas of rats[21]. Rats injected with Pho a showed a dramatic peak of Pho a in the ileum at the 6-8 h time point, suggesting that Pho might be concurrently found within the enterohepatic circulation.

Rabbit hepatic tissue contained Chl a, some Phe a and Phe b (apparently generated in the acidic environment of the stomach), as well as other metabolites, such as Chlide a and b, Pho and b. Chl a

was found in the liver throughout the study period and peaked at 8 h post-feeding, suggesting that the conversion of Chl a to CRCs was a slow process. Chlide a appeared in the liver at 2 h post-feeding and peaked at 8 h, which was consistent with the distribution of Chl a. Although Phe a was detected in hepatic tissue throughout the study period, Phe b and Phe b' were both undetectable in the liver at 8 h post-feeding, suggesting that Phe b was degraded to Pho b by removal of the phytyl group in the liver. The levels of Pho a and Pho b also peaked at 8 h and 4 h post-feeding, respectively, suggesting that hepatic Pho was not converted from Phe in the liver. Instead, it was possible that Chl a might be converted to Pho prior to absorption.

It was intriguing that Chlide a, Pho a and Pho b appeared earlier (2 h) in the gallbladder, compared to the liver (2 h *vs*. 8 h). Our data suggested that the behavior of CRCs in the enterohepatic circulation was similar to bile salts, which had a very low plasma concentration being rapidly taken up from the bloodstream by the liver[22]. Moreover, once taken up by the hepatocytes, bile salts are transported from the blood to the bile via special transporters[23]. Based on our data, we suggest that Chl a and b may be converted to Chlide a, Pho a and Pho by the enzymes or microflora in the gut, then absorbed by the ileum and transported through enterohepatic circulation, where they undergo rapid uptake by the liver and vectorial transport to the gallbladder. Although this mechanism explained the high levels of Pho found at 2 h in the gallbladder, it did not explain the lack of detectable hepatic Pho at that time.

In the present study, only Pho a and b were found in the blood and kidneys. Although Pho a and b were consistently observed in blood, their levels peaked at 12 h, which was 4 h later than the peak values found in the liver (8 h). Furthermore, the levels of renal Pho a peaked at 24 h, which was much later than that observed in the liver and bloodstream. These data suggested that Pho might be released by the liver, enter the peripheral blood circulation and then accumulate in the kidneys for excretion; however, further studies are necessary to confirm this.

We found somewhat similar levels of undigested Chl a and b throughout the period of feces collection (8 to 24 h). However, the levels of fecal Phe b were ten-fold higher compared to levels of Phe a, suggesting that Phe a might be more easily absorbed by the intestine. Although Phe a and b and their isomers were found in the feces, Chlide and Pho were not detected. These data suggested that CRCs might have transformed into Chlide and Pho, absorbed and utilized in those two forms by the gut microflora. Chlide is a water-soluble Chl derivative, which produces a variety of metallochlorophyllins through chelating metal ions, including Cu^{2+}, Fe^{2+} and Co^{2+}. Earlier, it has been shown to combine with cyclophosphamide to result in growth inhibition of colon cancer cells in mice[11] and suppression of aflatoxin (AFB1)-induced liver cancer in humans[24].

CRCs are important antioxidants found in daily foods. They reduce oxidation and have been shown to play a role in preventing H_2O_2-induced DNA damage, quenching 1,1-diphenyl-2-picrylhydrazyl (DPPH), chelation of Fe(II) and preventing formation of thiobarbituric acid reactive substances (TBARS) during Cu-mediated peroxidation of low density lipoprotein (LDL) in a dose-dependent manner[25,26]. CRCs have also been shown to downregulate the DNA-binding activity and expression of NF-$\kappa\beta$, p65 and AP-1 translocation in TNF α-treated HAECs, upregulate the DNA-binding activity and stimulate the TGF-β–induced SMAD3/4 signaling cascade in HASMCs and inhibit the DNA-binding activity and expressions of STAT3 in HASMCs[27].

In summary, our study demonstrated that Chlide and Pho were the principal Chl metabolites in the rabbits. Our data may expand our understanding of the metabolism and biodistribution of CRCs in the human body. As a number of biological functions, including anti-oxidation, anti-tumor and anti-aging have recently been attributed to CRCs, resulting in their wide use in food and medicine, it will be interesting to explore, if the binding of Chlide and Pho to other nutrients or trace metal ions in the body mediate their biological functions.

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