Assessment of Degradation and Intestinal Cell Uptake of Carotenoids and Chlorophyll Derivatives from Spinach Puree Using an In Vitro Digestion and Caco-2 Human Cell Model

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Although numerous studies have demonstrated the health benefits of chlorophyll derivatives, information regarding the digestion, absorption, and metabolism of these phytochemicals is quite limited. To better understand the digestion of these pigments, green vegetables including fresh spinach puree (FSP), heat- and acid-treated spinach puree (HASP), and $ZnCl_2$-treated spinach puree (ZnSP) were subjected to an in vitro digestion method which simulates both the gastric and small intestinal phases of the process. Native chlorophylls were converted to Mg-free pheophytin derivatives during digestion. Conversely, Zn-pheophytins were completely stable during the digestive process. Transfer of lipophilic chlorophyll derivatives, as well as the carotenoids lutein and β-carotene, into the aqueous micellar fraction from the food matrix was quantified. Micellarization of total chlorophyll derivatives differed significantly ($p < 0.05$) for FSP (37.6%), HASP (17.2%), and ZnSP (8.7%). Micellarization of chlorophyll *a* derivatives was determined to be significantly more efficient than chlorophyll *b* derivatives in FSP and HASP ($p < 0.01$), but not in ZnSP ($p > 0.05$). Intestinal cell uptake of micellarized pigments was investigated using HTB-37 (parent) and clonal TC7 lines of human Caco-2 cells. Medium containing the pigment-enriched fraction generated during digestion was added to the apical surface of fully differentiated monolayers for 4 h. Pigments were then extracted from cells and analyzed by C18 HPLC with photodiode array detection. Both Caco-2 HTB-37 and TC7 clone cells accumulated 20−40% and 5−10% of micellarized carotenoid and chlorophyll derivatives, respectively. These results are the first to demonstrate uptake of chlorophyll derivatives by human intestinal cells and to support the potential importance of chlorophylls as health-promoting phytochemicals.

Keywords: *Chlorophyll; lutein; β-carotene; in vitro digestion; Caco-2 cells; bioavailability*

INTRODUCTION

The health benefits associated with diets high in fruits and vegetables are well established (*1*−*4*). The rapid accumulation of data in recent years has strengthened the association between chemoprevention and consumption of foods rich in phytochemicals, including natural plant pigments (*5*, *6*). Chlorophylls are the most abundant plant pigment in nature, comprising as much as 1% of the dry weight of certain plant tissues (*7*). Sodium copper chlorophyllin, a commercial-grade water-soluble mixture of copper-chlorophyll derivatives, exhibits potent antimutagenic and antioxidant activities (*8*−*11*). Various studies have also focused on the natural chlorophylls prominent in the human diet. Lai et al. (*12*) showed a relationship between antimutagenic activity and chlorophyll content for a number of common vegetable extracts. More recently, in vitro and animal studies have shown that chlorophyll derivatives including chlorophyll *a*, pheophytin *a*, and pheophorbide *a* are potential chemopreventive agents (*10*, *11*, *13*−*15*). Likewise, pheophytin, pyropheophytin, and pheophorbide derivatives common to canned green vegetables were shown to have potent tumoricidal effects in vitro (*16*).

Information regarding the digestion, absorption, and metabolism of chlorophylls and their derivatives by humans remains limited. Several early studies focused on the isolation of chlorophyll derivatives in feces of humans and animals assuming negligible absorption (*17*−*19*). Because natural chlorophylls are modified by heat and acid, they may be susceptible to degradation when subjected to the harsh digestive environment (*20*−*23*). Select chlorophyll derivatives have been shown to exhibit different properties in in vitro tests (*16*, *23*). Therefore, a more complete understanding of the degree and nature of chlorophyll degradation through the gastrointestinal tract is important for the elucidation of the potential impact of this class of phytochemicals on human health.

The human intestinal cell line Caco-2 exhibits enterocyte-like characteristics (*24*) and its validity as a predictor of intestinal absorption in humans has been established for numerous lipophilic drugs (*25*, *26*). This model has also been utilized to investigate the uptake of nutrients and phytochemicals such as iron (*27*, *28*) and micellarized carotenoids (*29*, *30*). Recently, it was found that the TC7 clone of Caco-2 expresses 15-15′ dioxygenase activity (*31*). This cytoplasmic enzyme is responsible for cleavage of pro-vitamin A carotenoids,

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such as β-carotene into retinal, the precursor of vitamin A, or retinol (*32*).

The objectives of the present study were to investigate the modifications of natural chlorophylls during the gastric and small intestinal phases of digestion in vitro, and to characterize the uptake of micellarized chlorophyll and its degradation products by differentiated cultures of human intestinal Caco-2 cells. An understanding of these principles will expand our current understanding of the role these phytochemicals may play in human health.

MATERIALS AND METHODS

Chemicals and Standards. Extraction and chromatography solvents, methanol, ethyl acetate, petroleum ether, and water were of certified HPLC and ACS grade (Fisher Chemical, Fair Lawn, NJ). Pure standards of chlorophyll *a* and *b* were obtained from Sigma-Aldrich (St. Louis, MO). Pheophytin *a* and *b* standards were synthesized form chlorophyll *a* and *b* by as described by Schwartz et al. (*33*). Purity was confirmed by HPLC to be greater than 97%. Zn-pheophytin *a* and *b* were synthesized by as described by Schwartz (*34*). Zn-pheophytins were extracted with petroleum ether, separated, and isolated by HPLC to greater than 97% purity.

Sample Preparation. A spinach test meal was chosen for the study because of its high chlorophyll content. Spinach was purchased at a local market and homogenized in minimal deionized water to yield a puree. Lipid content of the homogenate was adjusted to 10% (wt/wt) by addition of corn oil, and the preparation was further homogenized to ensure even distribution of the oil and to increase stability of the final emulsion.

The spinach puree was subjected to heat, acid, and $ZnCl_2$ treatments to induce formation of prominent dietary chlorophyll derivatives including pheophytins and Zn-pheophytins (Figure 1). Approximately 1000 g of spinach puree was adjusted to 10% with corn oil and divided into aliquots of 300 g each. The first aliquot was frozen immediately at −80 °C (fresh spinach puree referred to as FSP). The second aliquot was acidified to pH 3 by addition of glacial acetic acid and heated to 100 °C for 20 min prior to freezing at −80 °C (heat- and acid-treated spinach puree referred to as HASP). The final aliquot was heated for 20 min at 100 °C with an excess of crystalline $ZnCl_2$ ($ZnCl_2$-treated spinach puree referred to as ZnSP).

In Vitro Digestion. Simulated digestions were performed as described by Garret et al. (*29*). Briefly, representative aliquots of homogenized test meals were subjected to a two-phase in vitro digestion process designed to mimic the in vivo process. The gastric phase included acidification of the sample to pH 2 with 100 mM HCl and treatment with porcine pepsin (3 mg/mL) with incubation at 37 °C for 1 h in a shaking water bath. The intestinal phase was initiated by neutralization with $NaHCO_3$; addition of porcine pancreatin (0.4 mg/mL), lipase (0.2 mg/mL), and porcine bile extract (2.4 mg/mL); adjustment of final pH to 7 with 1 N NaOH; and incubation at 37 °C for 2 h in a shaking water bath. Upon completion of the phases, aliquots of the digesta were centrifuged at 167 000*g* at 4 °C for 35 min (Beckman model L7-65 Ultracentrifuge) to separate the aqueous micellar phase from the residual solid and oil. The aqueous fraction was filtered (0.2 micron pore size) to remove any contaminating nonmicellarized aggregates. All procedures were performed in subdued light and samples were blanketed with nitrogen to minimize oxidation. Aliquots of raw materials, digesta, and aqueous micellar fraction were collected and stored at −80 °C until analysis.

Cellular Uptake of Micellarized Chlorophyll Derivatives and Carotenoids. Studies for chlorophyll derivatives used the Caco-2 cellular model as described by Garrett et al. (*29*). The parent line of Caco-2 (HTB 37) was obtained from the American Type Cell Culture Collection (Rockville, MD) and the TC7 clone of Caco-2 was kindly provided by Dr. Monique

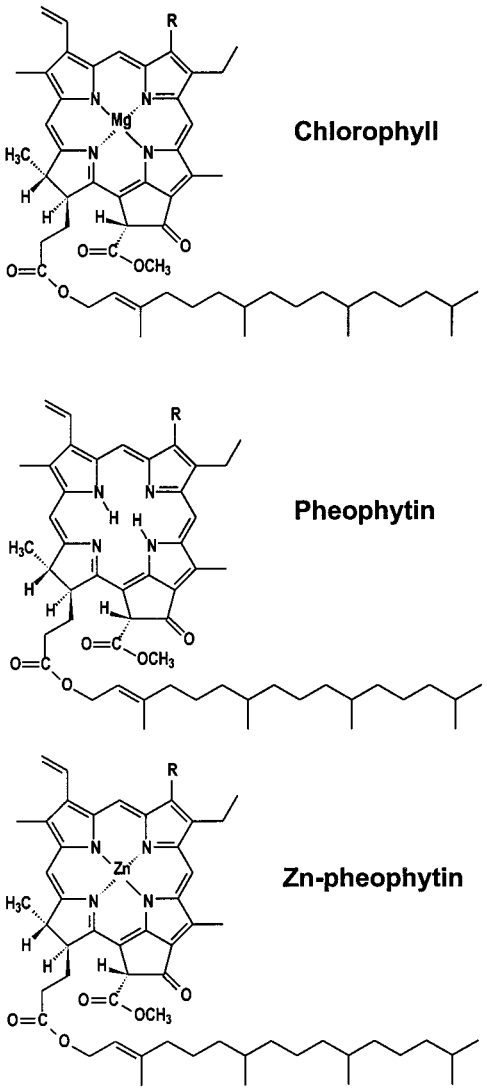

Figure 1. Structures of three main chlorophyll derivatives from fresh spinach puree (FSP), heat- and acid-treated spinach puree (HASP), and $ZnCl_2$-treated spinach puree (ZnSP). For *a* derivatives R = CH_3; for *b* derivatives R = CHO.

Rousset, INERM, Cedex, France. Stocks were maintained as described by Han et al. (*27*) with complete medium containing high-glucose DMEM (Sigma, St. Louis, MO), 10.0% heat inactivated fetal bovine serum, nonessential amino acids (10 mL/L), L-glutamine (2.0 mmol/L), amphotericin B (0.5 mg/L), gentamicin (50 mg/L), HEPES (15 mmol/L), and sodium bicarbonate (44 mmol/L). Test cells were grown and differentiated in 12-well plastic dishes in a humidified atmosphere of air/CO_2 (95:5) at 37 °C. All experiments used highly differentiated monolayers at passages 22−35 and 75−80 for HTB 37 and TC7, respectively, 11−14 days after reaching confluency. Prior to initiating the uptake experiment, monolayers were washed twice with 1 mL of Hank's balanced salt solution. Monolayers were exposed to filtered aqueous fraction from the in vitro digestion diluted 1:3 with basal DMEM. Cultures were incubated for 4 h at 37 °C. The test media was removed and the monolayers were washed twice with ice-cold Hank's balanced salts solution containing 5.0 mmol/L sodium taurocholate. Cells were collected in 1.0 mL of ice-cold phosphate buffered saline containing 10.0% ethanol and 45 μmol BHT and stored at −80 °C.

Raw Material Extraction. Lipophilic carotenoids and chlorophyll derivatives were extracted from 5 to 20 g of raw material by homogenization in acetone with added $CaCO_3$ and subsequent vacuum filtration (repeated 3×). Filtrates were combined in a separatory funnel and mixed with 30 mL of petroleum ether. Deionized water was then added to partition

the chlorophyll derivatives into the petroleum ether phase which was collected and diluted volumetrically to 50 mL. Aliquots were removed and dried under a stream of nitrogen gas. Dried samples were then dissolved in 2−4 mL of acetone in preparation for LC analysis.

Digesta and Aqueous Micellar Fraction Extraction. Carotenoids and chlorophyll derivatives were extracted from a 1−4.0 mL sample by addition of 4.0 mL acetone/petroleum ether (50:50) (0.1% BHT) and vortexed for 1 min. The sample was then centrifuged (2000*g*) for 2 min to hasten phase separation. The petroleum ether layer was collected and saved. Extraction was repeated a total of 3 times and the combined petroleum ether fractions were dried under a stream of nitrogen gas, redissolved in acetone, and analyzed immediately.

Caco-2 Cell Extractions. Pigment extraction from Caco-2 cells was performed using a method modified from Peng and Peng (*35*). Briefly, 200 μL of protease solution (100 mg protease/10 mL PBS) was added to each cell pellet. Following incubation of the pellets at 37 °C for 30 min 0.5 mL of SDS-EtOH (0.1% BHT) solution was added, and the sample was vortexed for 1 min. Chlorophyll derivatives were extracted by addition of 0.5 mL of acetone/petroleum ether (1:2). The samples were vortexed and briefly centrifuged (2000*g*) to hasten the phase separation. The petroleum ether layer was collected and saved. The extraction was repeated a total of three times and the combined petroleum ether fractions were dried under a stream of nitrogen and analyzed immediately.

Chromatography. High performance liquid chromatography (HPLC) analysis of chlorophyll derivatives was performed by the method of Schwartz et al. (*33*) with modification. A Waters 2690 Separations Module (Milford, MA) with a model 998 photodiode array detector was used for analysis. A Vydac 201TP54 analytical scale (4.6 mm i.d. × 150 mm) reversed-phase column (Hesperia, CA) with a C18 stationary-phase guard column was used.

Separations were achieved using a gradient elution with a binary mobile phase of methanol−water in reservoir A (75:25) and ethyl acetate in reservoir B. Initial conditions were set at 100% A with a linear gradient to 50:50 A/B over 10 min. The gradient was held for 10 min before following a 5.0 min linear gradient back to 100% A for a final chromatographic run time of 30 min. Detection and tentative identification of all chlorophyll derivatives was accomplished using in-line photodiode array data between 350 and 700 nm.

Data Analysis. The percent of lipophilic derivatives transferred from the raw material food matrix into the aqueous micellar fraction is defined as the micellarization percentage (%M). All data were analyzed using StatView 5.0 (SAS Institute, Cary, NC). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each pigment's extent of micellarization and concentration in Caco-2 cells. Group differences were determined by analysis of variance using Fisher's PLSD post-hoc test ($\alpha < 0.05$).

RESULTS AND DISCUSSION

HPLC Analysis. Representative LC chromatograms for FSP, HASP, and ZnSP are shown in Figure 2. Separation of six major chlorophyll derivatives is illustrated including predominant native chlorophyll *a* and *b*, pheophytin *a* and *b*, and Zn-pheophytin *a* and *b*. Further separation of corresponding allomers and epimers was also achieved (data not shown). The composition of the three starting materials was indicative of the method of preparative treatment. FSP contained chlorophylls *a* and *b* in a 2.5:1 ratio (Table 1) which is consistent with published literature values (*22*, *33*). In contrast, HASP contained the Mg-free chlorophyll derivatives pheophytin *a*, *a*′, *b*, and *b*′. Pheophytin epimers represented approximately 15% of the total pheophytin (Table 1). Mg-free pheophytin derivatives are known to be formed through commercial processing

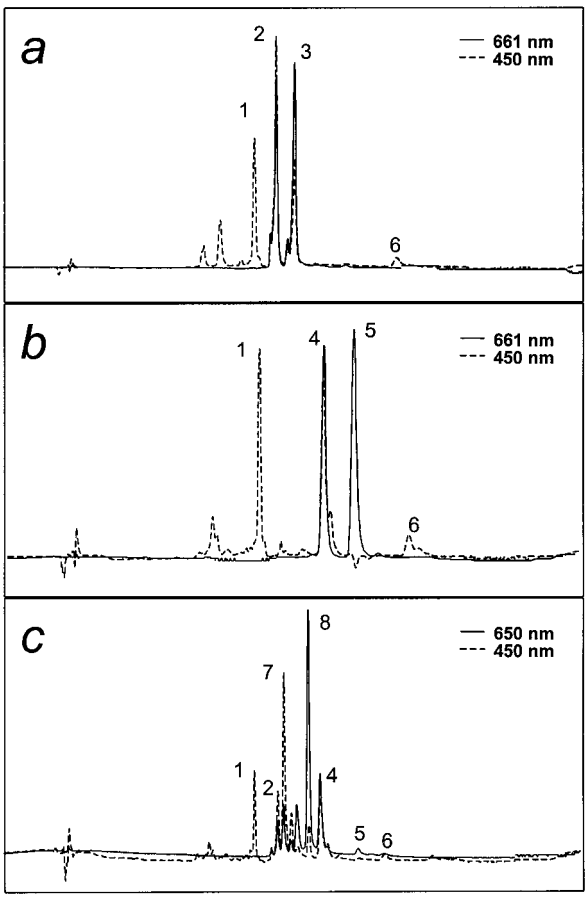

Figure 2. Representative HPLC chromatograms of chlorophyll and carotenoid derivatives present in (a) fresh spinach puree (FSP), (b) heat- and acid-treated spinach puree (HASP), and (c) $ZnCl_2$-treated spinach puree (ZnSP). The preparation of these spinach-based meals is described in Materials and Methods. Peak identifications: 1 = lutein; 2 = Chlorophyll *b*; 3 = Chlorophyll *a*; 4 = Pheophytin *b*; 5 = β-carotene; 6 = Pheophytin *a*; 7 = Zn−Pheophytin *b*; 8 = Zn−Pheophytin *a*.

conditions (*33*). ZnSP contained Zn-pheophytin *a*, *a*′, *b*, *b*′, pheophytin *b*, and chlorophyll *b*. Residual chlorophyll *b* and pheophytin *b* in this preparation are expected because the formation of Zn-pheophytin *b* complexes is known to be poor (*36*). This has been attributed to both a lower concentration of the parent compound (pheophytin *b*) in the vegetable tissue and the presence of the electron-withdrawing formyl group lowering electron density that decreases its reactivity with metal ions (*21*). The quantities of the major carotenoids, lutein and β-carotene, were not altered by the preparative treatments.

Chlorophyll Degradation. The impact of both gastric and small-intestinal phases of digestion on dietary chlorophylls was examined. Although the profile of chlorophyll derivatives in FSP and HASP raw material differed, distribution in the digesta of FSP and HASP were similar (Table 1). Approximately 75−77% of total chlorophyll pigments for both meals were pheophytin *a* derivatives after digestion. Elevated levels of allomerized pheophytin *a* and *b* were present after digestion of FSP, but not after digestion of HASP. Allomerized derivatives are hydroxy-chlorophylls formed through oxidation of parent chlorophylls with addition of oxygen to the isocyclic ring (*22*). It is unclear whether the oxidation occurs prior to or after loss of the central magnesium atom. However, the low levels of allomer-

Table 1. Relative Amounts of Chlorophylls and Pheophytins in Raw Material, Digesta, and Aqueous Micellar Fraction for Fresh Spinach Puree (FSP), Heat- and Acid-Treated Spinach Puree (HASP), and $ZnCl_2$ Spinach Puree (ZnSP)[a]

	FSP			HASP			ZnSP		
	raw material	digesta	aqueous micellar fraction	raw material	digesta	aqueous micellar fraction	raw material	digesta	aqueous micellar fraction
CHL a	71.0								
CHL b	29.0						5.0		
PHE a		50.0	66.0	65.0	65.0	67.0			
PHE a′		12.0	14.0	10.0	9.0	15.0			
PHE a(ox)		16.0			1.0				
PHE b		15.0	17.0	22.0	23.0	15.0	4.0	4.0	
PHE b′		2.0	3.0	3.0	2.0	3.0			
PHE b(ox)		5.0							
ZnPHE a							66.0	64.0	70.0
ZnPHE a′							12.0	13.0	15.0
ZnPHE b							10.0	13.0	10.0
ZnPHE b′							3.0	6.0	5.0

[a] Raw materials were subjected to the standard digestion procedures described in Materials and Methods. Data are presented as percent of total and represent an average of 5 independent measurements. Abbreviations: CHL, chlorophyll; PHE, pheophytin; PHE′, pheophytin epimer; PHE (ox), allomerized pheophytin; ZnPHE, Zn-pheophytin.

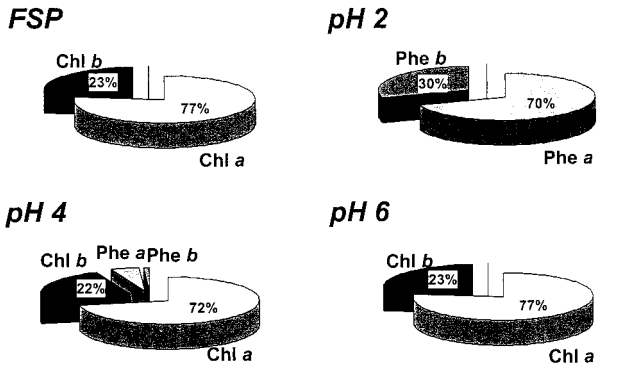

Figure 3. Influence of gastric pH on conversion of chlorophyll to pheophytin during the gastric phase of digestion. Raw material composition prior to digestion is indicated as FSP. Gastric pH was adjusted to experimental conditions with 1.0 N HCl prior to addition of pepsin. After incubation at 37 °C for 1 h in a shaking water bath, samples were neutralized (pH 7) by addition of 1.0 M $NaHCO_3$ followed by titration with 1.0 N NaOH. Analysis was performed as described in Materials and Methods. Values represent an average of four independent gastric digestions.

ized pheophytin in HASP points to oxidation of chlorophyll in FSP prior to pheophytin formation during digestion.

The standard conditions for the gastric phase of in vitro digestion are incubation at 37 °C in a shaking bath at pH 2 for 1 h. The sensitivity of chlorophyll derivatives to acidity is well established (*21*). Numerous factors including age, physiological conditions, over the counter antacids, and prescription H2-receptor antagonist have been shown to affect human gastric pH (*37*−*39*). For this reason the impact of elevated pH on the conversion efficiency of chlorophylls to their respective pheophytins in FSP was investigated. There was minimal loss of the central Mg from the chlorophyll porphyrin (6.0%) at pH 4, and the native chlorophylls were stable during incubation at pH 6 for 1 h (Figure 3). Therefore, both native chlorophylls and pheophytins may be present in the gastrointestinal tract of humans with elevated gastric pH. In addition, conversion of chlorophylls *a* and *b* to their respective pheophytins was complete after incubation at pH 2.0 for 0.5 h with greater than 95% of initial chlorophyll content from FSP recovered as pheophytin in the digesta (data not shown). The above data suggest that the cleavage of the phytol tail to produce pheophorbides is unlikely to occur during the gastric and small intestinal phases of digestion. Thus, isolation of pheophorbides in human feces most likely resulted from subsequent metabolism by intestinal microflora (*7*).

Regardless of initial food treatment, the aqueous micellar composition for both FSP and HASP samples was very similar with major components being pheophytin *a*, *a*′, *b*, and *b*′ (Table 1). The profile of the aqueous fraction provides insight about which lipophilic chlorophyll derivatives may be micellarized for uptake and possible transport by intestinal epithelial cells (*40*).

In contrast to the native chlorophylls from FSP, the Zn-pheophytins from the ZnSP were completely stable during in vitro digestion (Table 1). The small amount of chlorophyll *b* (5.0%) found in the raw material was most likely degraded to pheophytin *b* through the simulated digestion. The absence of pheophytin *b* in the final aqueous micellar fraction may be explained by the poor association of the metal-free derivatives with lipid micelles and will be discussed below. Commercial products may contain Zn-pheophytin and pyropheophytin derivatives formed through a trademarked process known as Veri-Green (*41*). The stability of these dietary chlorophylls when exposed to digestive conditions is intriguing because modulation of chlorophyll composition in the gastrointestinal tract may affect the transport and function of these phytochemicals in humans.

Micellarization of Chlorophyll Derivatives. The efficiency of transfer of the lipophilic chlorophyll derivatives from the food matrix to the aqueous fraction during the two-phase digestion process is defined as percent micellarization (%M). The percentages of chlorophyll derivatives that were micellarized during digestion of the three spinach test meals are shown in Figure 4a. These data represent total chlorophyll, the sum of natural chlorophylls, and their corresponding Mg-free pheophytin derivatives previously described in Table 1. Micellarization of chlorophyll derivatives was significantly more efficient from FSP (37.6%) than either HASP (17.2%; $p < 0.01$) or ZnSP (8.7%; $p < 0.001$). Omission of bile extract from the standard digestion prevented transfer of chlorophyll derivatives to the aqueous fraction, confirming their association with lipid micelles (data not shown).

The decreased efficiency of micellarization of chlorophyll derivatives in digested HASP and ZnSP may be due to the effects of processing on the digestibility of the vegetable tissue. Components formed or released during thermal processing, acidification, or $ZnCl_2$ treat-

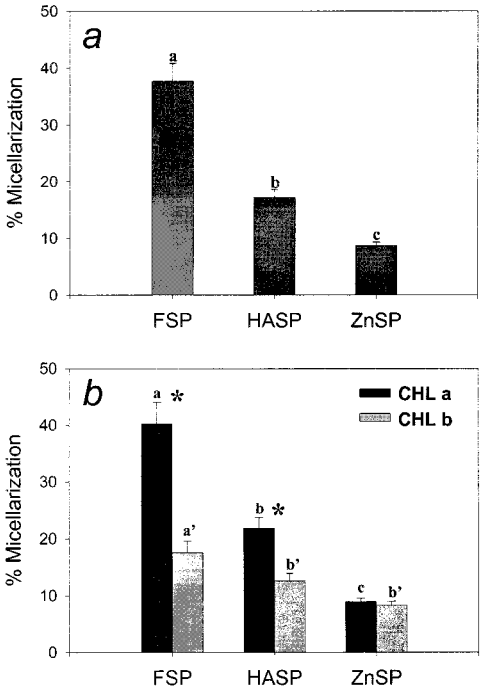

Figure 4. Micellarization of chlorophyll derivatives from test meals. (a) Percent micellarization of total chlorophyll (CHL = sum of residual chlorophylls and pheophytins) from FSP, HASP, and ZnSP spinach test meals. (b) Comparison of CHL *a* and *b* derivatives from digested FSP, HASP, and ZnSP test meals. Data represent mean ± SEM for 5 independent digestions. The presence of different letters over the error bars indicates that micellarization of both total chlorophyll and chlorophyll *a* and *b* differed significantly between test meals ($p < 0.05$) with the exception of chlorophyll *b* derivatives between HASP and ZnSP where the difference was determined to be statistically insignificant ($p > 0.05$). The presence of the asterisk (*) above the bars indicates those test meals in which micellarization of chlorophyll *a* and *b* derivatives differed significantly ($p < 0.05$); FSP ($p < 0.001$), HASP ($p < 0.05$); ZnSP ($p > 0.05$).

ment may impair digestion enzyme activities or alter micelle formation. For example, specific food components such as minerals and fiber have been shown to interact with bile salts (*42*, *43*). Excess zinc has been reported to limit activity of select pancreatic enzymes (*44*). Chlorophyll derivatives have also been shown to hinder pancreatic enzyme catalytic capacity. This effect has been postulated to be a result of binding of the porphyrin backbone to proteolytic enzymes (*45*). Also, the central zinc atom of Zn-pheophytins maintains a coordination number of six. This allows the metal to participate in two interactions distinct from the four secondary valence bonds to the porphyrin backbone. It is possible that formation of coordinate covalent interactions between zinc near functional groups on the amino acid residues of enzymes may impair normal digestive events and lead to inefficient micellarization.

The relative efficiency of micellarization of chlorophyll *a* and *b* derivatives (i.e., residual native chlorophylls and pheophytins for each class) also was compared for the three test meals (Figure 4b). Micellarization of chlorophyll *a* derivatives was significantly greater than that of *b* derivatives for FSP and HASP ($p < 0.01$), but not for ZnSP ($p > 0.05$). Although micellarization of chlorophyll *b* derivatives was significantly higher ($p < 0.05$) for FSP than for HASP, the efficiency of micellarization remained only minimally affected by pre-digestion treatment ranging from 9.0−18%. Conversely, micellarization of chlorophyll *a* derivatives was strongly affected by the pre-digestion treatment. Micellarization of chlorophyll *a* derivatives was 40.0, 22.0, and 10.0% for FSP, HASP, and ZnSP, respectively. This profile closely matched the overall trend seen in Figure 4a and demonstrates that the fate of the more abundant chlorophyll *a* derivatives contributes markedly to the profile of total chlorophyll micellarization.

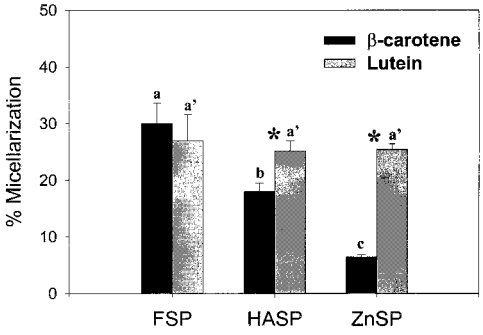

Figure 5. Comparison of lutein and β-carotene micellarization from digested FSP, HASP, and ZnSP test meals. Data represent mean ± SEM for 5−9 independent digestions for each test meal. The presence of different letters over the error bars indicates that mean β-carotene micellarization differed significantly ($p < 0.05$) between test meals. Differences in mean micellarization of lutein between test meals were determined to be statistically insignificant ($p > 0.05$). The presence of the asterisk (*) above the bars indicates those test meals in which micellarization of lutein and β-carotene derivatives differed significantly ($p < 0.05$); HASP ($p < 0.05$), ZnSP ($p < 0.001$), FSP ($p > 0.05$).

The significant difference in micellarization between chlorophyll *a* and *b* derivatives may be explained by consideration of structural differences between the groups of chlorophyll (*a* and *b*). The presence of a formyl group increases the hydrophilicity of chlorophyll *b* derivatives relative to chlorophyll *a* derivatives (*22*), which is evident by the more rapid elution of chlorophyll *b* derivatives on the reverse-phase chromatography system employed in this study (Figure 2). The more hydrophobic chlorophyll *a* derivatives may be more efficiently micellarized because of more optimal solubility in the mixed micelle. This possibility is supported by our observations of the micellarization of the carotenoids lutein and β-carotene in the digested spinach samples. The efficiency of micellarization for lutein and β-carotene is depicted in Figure 5. Whereas micellarization of polar lutein was not significantly affected by type of treatment, there was a significant reduction in micellarization of apolar β-carotene in response to the digestion of HASP ($p < 0.05$) and ZnSP ($p < 0.001$).

Caco-2 Cellular Uptake. The uptake of chlorophyll derivatives by human intestinal cells was studied to validate the availability of micellarized chlorophyll derivatives. Studies were performed using fully differentiated cultures of both the HTB-37 (parent) line and the TC7 clone of Caco-2 cells. Aqueous micellar fractions of each test meal were collected and diluted 4-fold with DMEM for presentation to monolayers. Cellular content was determined for spinach chlorophylls and carotenoids after 4 h. As with digesta and aqueous micelle fractions of both FSP and HASP, pheophytins *a*, *a′*, *b*, and *b′* were the predominant chlorophyll derivatives in the Caco-2 cells. The total amounts of chlorophyll *a* and *b* derivatives and carotenoids from FSP and HASP in both HTB-37 and TC7 cell lines are shown in Figure 6a and 6b. Cells ac-

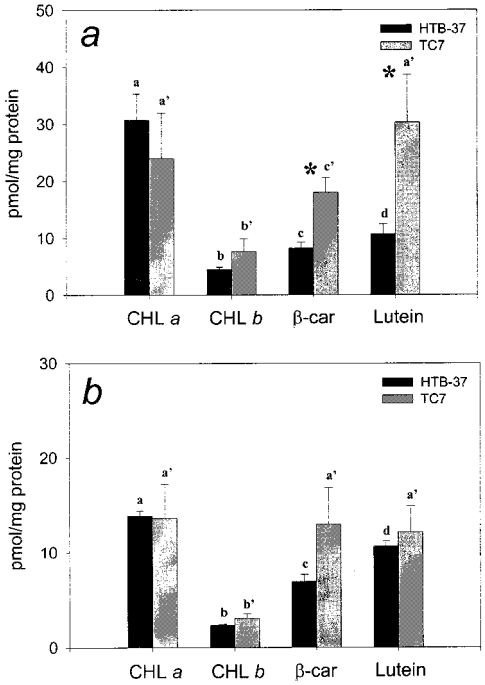

Figure 6. Caco-2 cellular content (pmol/mg protein) of micellarized chlorophyll and carotenoid pigments generated from the digestion of (a) fresh spinach puree (FSP); (b) heat- and acid-treated spinach puree (HASP). Data represent mean quantity of pigment present per mg of cell protein after 4 h incubation in medium containing aliquots of aqueous (micellar) fraction of the digestate. CHL = sum of residual native chlorophylls and pheophytins. Data represent mean ± SEM for 3−4 independent measurements. The presence of different letters indicates mean cellular accumulation of each pigment differed significantly ($p < 0.05$) within both the HTB-37 parent and TC7 clonal lines of Caco-2. The presence of the asterisk (*) above the bars for β-carotene and lutein in panel (a) indicate that the uptake of these carotenoids by the HTB-37 parent line and TC7 clonal lines of Caco-2 differed significantly ($p < 0.05$).

cumulated 3−30 pmol of chlorophyll *a* and *b* derivatives per mg of cellular protein. Cells also accumulated between 10 and 30 pmol of lutein per mg of cell protein and 6−20 pmol of β-carotene per mg of cell protein. Cellular accumulation of chlorophyll derivatives represented 5−10% of the amount of micellarized chlorophyll *a* and *b* derivatives in the medium compared to 20−40% uptake of micellarized carotenoids. The extent of carotenoid uptake is similar to that previously reported by Garrett et al. (*29*). The lower pigment content of cells incubated with HASP test media resulted from depressed micellarization of pigments from HASP during simulated digestion (Figure 4a, b). These findings demonstrate that cellular content is proportional to micellar content and accentuate the importance of efficient micellarization for the maximization of cellular concentration of these lipophilic phytochemicals.

Two-way analysis of variance showed a significant interaction ($p < 0.05$) between cell line and test meal for lutein only. A main effect of test meal was seen for lutein, chlorophyll *a*, and chlorophyll *b* derivatives with mean cellular content of these pigments consistently higher after exposure to diluted micellar fraction from digested FSP for the reasons described above. The failure to detect Zn-pheophytins in Caco-2 cells is attributed to the low degree of micellarization of these chlorophyll derivatives from the digested ZnSP. Assuming that the monolayers accumulated 5−10% Zn-pheophytins as observed with pheophytin derivatives from aqueous micelle fraction of both FSP and HASP, the employed methodology lacked sufficient sensitivity to confidently detect and identify these analytes.

Relative accumulation of chlorophyll derivatives was similar for both HTB-37 and TC7 Caco-2 cells ($p > 0.05$). In contrast, cellular accumulation of β-carotene and lutein by the TC7 clonal line was significantly higher than that by HTB-37 cells ($p < 0.05$). This initial observation was surprising because 15−15′ dioxygenase activity has been reported in the TC7 clone (*31*) but is not present in the HTB-37 line of Caco-2. Although the metabolism of spinach carotenoids by these cells was not investigated, the greater accumulation might represent more efficient uptake or retention of carotenoids by this clonal line.

The lower cellular accumulation efficiency of chlorophyll derivatives compared to that of carotenoids may be a result of specific physiochemical properties of chlorophyll. Parameters such as hydrophobicity, ionization, and molecular size have been strongly related with differential intestinal absorption (*46*). In model systems such as rat intestinal perfusion, rat intestinal rings, and Caco-2 cells, an increase in molecular weight has been correlated with lower intestinal permeability (*46*). Chlorophyll content in spinach can be as high as 1500 ppm on a wet weight basis compared to 200 ppm for carotenoids (*22*). Therefore, it is likely that enterocytes can accumulate significant amounts of chlorophyll derivatives after ingestion of meals containing green vegetables. The possibility of subsequent cellular metabolism and transport of these compounds merits investigation.

In conclusion, pheophytins and their epimers were micellarized from spinach test meals subjected to in vitro digestion. Although Zn-pheophytins were stable during the simulated gastric and small intestinal phases of digestion, native chlorophylls degraded rapidly to pheophytins in response to the high acidity of the gastric phase. These results indicate that it is possible for multiple chlorophyll derivatives to coexist within mixed micelles. The presence of chlorophyll derivatives in the micelle fraction after digestion and accumulation by Caco-2 enterocyte-like cells suggests that these compounds or their metabolites may be absorbed and transported to peripheral tissues. Furthermore, the absence of chlorophyll *a* and *b* in the digesta and aqueous micellar fractions when the pH of the gastric phase of digestion was low (pH 2) indicates that pheophytins are abundant and likely represents the biologically relevant forms of these natural pigments in most humans.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; FSP, fresh spinach puree; HASP, heat- and acid-treated spinach puree; ZnSP, $ZnCl_2$-treated spinach puree; DMEM, Dulbecco's modified eagles medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PBS, phosphate buffered saline; BHT, butylated hydroxytoluene.

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