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Determination of the Concentration of Potential Efflux Pump Inhibitors, Pheophorbide *a* and Pyropheophorbide *a*, in the Feces of Animals by Fluorescence Spectroscopy

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ABSTRACT: Efflux pumps are vital bacterial components, and research has demonstrated that some plant compounds such as pheophorbide *a* (php) possess efflux pump inhibitor (EPI) activity. This study determined the quantity of php present in feces as an indicator of EPI activity. Feces were collected from different species of animals fed a variety of feeds. The chlorophyll metabolites php and pyropheophorbide *a* (pyp) were determined using fluorescense spectroscopy. The average concentrations [μg/g dry matter (DM) feces] of pyp/php in feces were as follows: guinea pig, 180; goat, 150; rabbit, 150; dairy cow, 120; feedlot cattle, 60; rat, <1; pig, <1; chicken, <1. These data indicate that animals consuming "green" diets will excrete feces with concentrations of php/pyp that exceed levels demonstrated to be inhibitory to bacterial efflux pumps (0.5 μg/mL). The natural presence EPIs in the gastrointestinal tract may modulate the activity of microbial efflux pumps and exert selection pressure upon resident microbial populations.

KEYWORDS: *efflux pump inhibitor, pheophorbide a, pyropheophorbide a, feces, multidrug resistance*

INTRODUCTION

Since their discovery in the early 1980s, bacterial multidrug resistance (MDR) efflux pumps have been extensively investigated in regard to antimicrobial resistance.[1] MDR efflux pumps appear to be involved in bacterial resistance to a wide range of antibiotics, and transport capabilities have been reported for many classes of efflux pumps.[2] Evidence exists suggesting that these pumps may be ubiquitous and possess other functions important to microbial metabolism.[3,4] Although their description is incomplete, it is clear that these pumps are an important mechanism of antimicrobial resistance.[5] All require energy to function, with some types using ATP directly and others depending upon proton or sodium gradients.[6] Some of the best described pumps for bacteria include the AcrAB-TolC pump in *Escherichia coli*, MexABOprM in *Pseudomonas aeruginosa*, and FloR in *Salmonella enterica* serovar Typhimurium.

Research concerning the activity of these pumps indicates that they can be inhibited by a variety of compounds that render them nonfunctional in terms of antibiotic efflux.[7] Therefore, by denying a microbe the means to remove accumulated antibiotics from within the cell, these inhibitors are seen as potential adjuvants to reverse antimicrobial resistance in a manner similar to the role played by clavulanic acid as a β-lactamase inhibitor.[7,8] Whereas the search for inhibitors is primarily an effort focused on clinical applications,[9] the role of food and diet is also of interest, and some secondary compounds from plants are reported to have efflux pump inhibiting activity.[10−12]

Some of the first evidence that natural plant compounds are inhibitors of efflux pumps was reported by the Stermitz and Lewis group in 2000.[13,14] In this work, they identified and isolated a flavolignan (5′-methoxyhydnocarpin) from extracts of *Berberis trifoliolata* and demonstrated its inhibition of the NorA efflux pump of *Staphylococcus aureus*. They subsequently identified pheophorbide *a*, a chlorophyll metabolite, as an even more potent efflux pump inhibitor using the same NorA assay.[15] In later work, they screened the inhibitory activity of plant extracts against several bacteria including *Bacillus megaterium*, *E. coli*, *S. enterica*, and *P. aeruginosa*. The most potent inhibitors lowered the minimum inhibitory concentration (MICs) for several of the antibiotics tested 10−100-fold.[11] From an evolutionary perspective, these authors concluded that plant-derived efflux pump inhibitors allowed antimicrobials within the plant, which when alone do not show strong antimicrobial activity, to be more effective against bacterial pathogens. Additionally, the pump-inhibiting activity of some plant compounds could be considered to be part of a complex mechanism of defense against microbial pathogens. One example highlighted by Tegos et al. was that of rhein, an anthraquinone from rhubarb.[11] This compound alone had essentially no activity against *Pseudomonas* (MIC > 500 μg/mL) but when assayed with one of several efflux pump inhibitors, the MIC of rhein was reduced 100-fold (5 μg/mL). Similarly, Musumeci et al. reported large MIC reductions when pheophorbide was used to block the pumps responsible for ciprofloxacin efflux in resistant strains of *S. aureus*.[16]

The activity of pheophorbide *a* as an efflux pump inhibitor is one of particular interest in our investigations. Degradation of chlorophyll in the anaerobic environment of the gut follows a mechanism dissimilar to the aerobic metabolism within leaf

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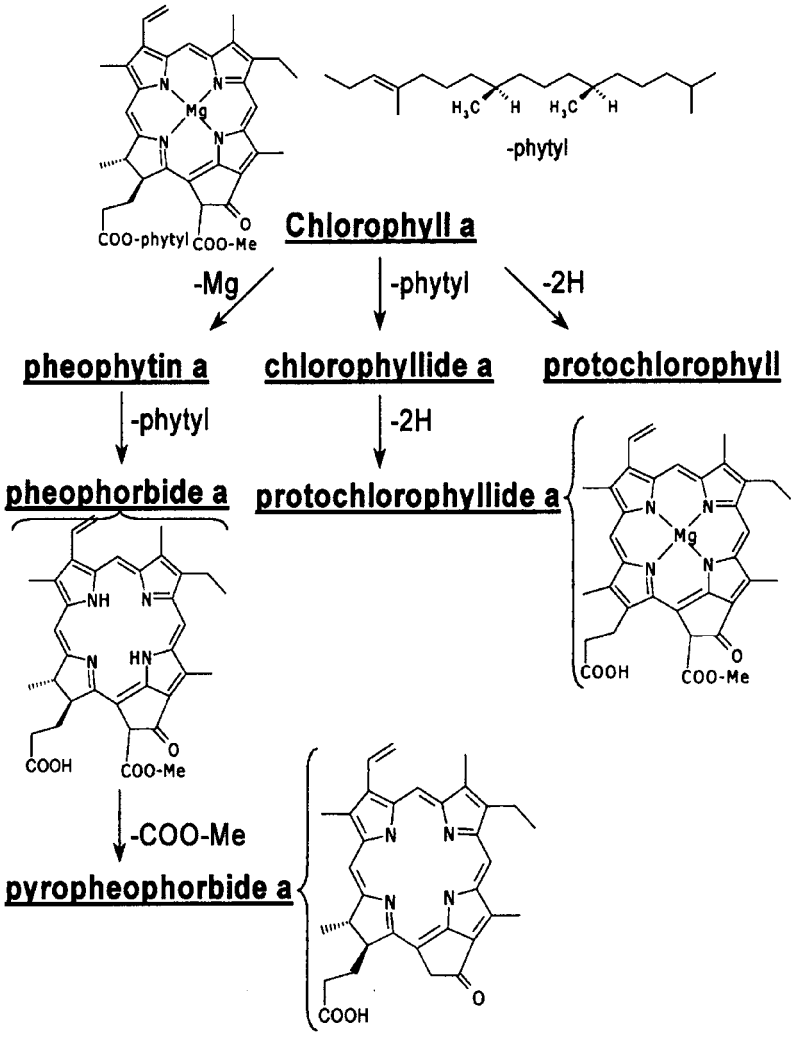

Figure 1. Metabolism of chlorophyll into various metabolites in the gastrointestinal tract. Reproduced from reference 21, copyright 2003, American Chemical Society.

tissue. Chlorophyll turnover in plants occurs when the porphyrin ring is oxidatively degraded into a linear tetrapyrrole.[17,18] In contrast, anaerobic metabolism in the gut leaves the porphyrin ring intact (Figure 1). As a result of these differences, chlorophyll degradation in the gut produces large quantities of pheophorbide *a*, which may have significant inhibitory activity on efflux pumps of enteric bacteria. On the basis of this background information, the work presented here was designed to determine the concentration of chlorophyll metabolites in the feces of animals.

MATERIALS AND METHODS

Freshly voided feces were collected from several individuals ($n > 3$) of different species of laboratory and farm animals being fed a wide variety of animal feeds (Table 1). Samples were dried at 60 °C in the dark. Fifty milligrams of dried fecal material was finely ground and added to 5.0 mL of 2-propanol (Sigma-Aldrich, >99.9%). Overnight extraction and sedimentation at 4 °C was allowed before fluorescence measurements were collected. Preliminary experiments indicated that overnight extraction resulted in extraction coefficients exceeding 95%. Fluorescence spectra were obtained on a SPEX Fluoromax-4 spectrofluorometer (ISA Jobin-Yvon/SPEX, Edison, NJ, USA) with a 1 nm band-pass and corrected for lamp spectral intensity and detector response. Fecal extracts were excited at λ_{ex} = 420 nm with an interference filter on the excitation side, and emission was collected at

Table 1. Animal Diet Composition

animal type	primary feed ingredients	relative chlorophyll content of diet
guinea pig	alfalfa meal	high
goat	alfalfa hay, other grass hay	high
rabbit	alfalfa meal	high
dairy cow	fresh orchard grass, corn grain	high
feedlot cattle	corn grain, corn silage	medium
pig	corn grain, soybean meal	low
rat	corn, wheat, soybean meal	low
chicken	corn, other grain, soybean meal	low

$\lambda_{em} \geq 430$ nm using a cutoff filter before the detector to eliminate scattered light. A calibration curve was generated with equal quantities of authentic standards of pheophorbide *a* and pyropheophorbide *a* (Frontier Scientific, Logan, UT, USA) in 2-propanol using peak emission at λ_{em} = 673 nm. Measurements on samples from each individual animal within a species were performed in triplicate.

RESULTS AND DISCUSSION

The intact chlorophyll present in the chloroplasts of plants is weakly fluorescent as 98−99% of the light energy absorbed is

efficiently transferred to other molecules during the normal process of photosynthesis.[19] However, in the gastrointestinal (GI) tract, chlorophyll is degraded into various metabolites.[20] As a result, the chlorophyll metabolites become much more fluorescent. Although other metabolites may also be present in the feces of animals, previous spectroscopic work has demonstrated that pheophorbide *a* (php) and pyropheophorbide *a* (pyp) are the two predominant compounds.[20−22] Thus, the feces of animals whose diets are chlorophyll-rich give a strong fluorescence signal corresponding predominately to php and pyp. Previously these optical properties have been used to develop instrumentation for the sensitive detection of fecal material on the meat of carcasses.[21] In the current work we utilize these optical characteristics to determine the amount of php and pyp in the feces of various species of animals on diets that vary in the amount of chlorophyll-containing components.

The fluorescence spectra obtained when 1 μM solutions of php and pyp were determined (λ_{ex} = 420 nm) are very similar, and both compounds displayed a maximum emission peak at λ_{em} = 673 nm. The pyp/php spectral ratio was ∼1.3 (Figure 2).

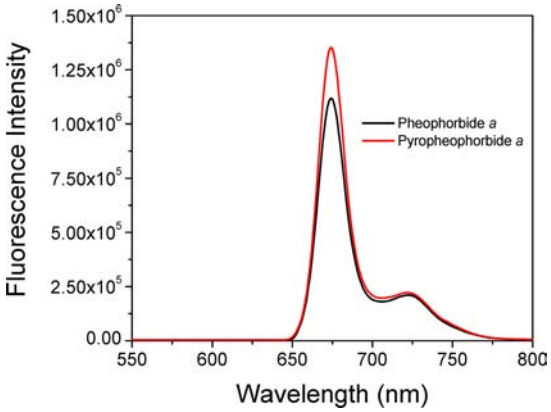

Figure 2. Fluorescence spectra of pheophorbide *a* (1 μM) and pyropheophorbide *a* (1 μM) in 2-propanol (λ_{ex} = 420 nm). The ratio of peak fluorescence intensity was ∼1.3 pyp/php.

Because it is not possible to resolve these two on the basis of their fluorescence spectra, we assumed a 50/50 mix of these two in the feces of animals, for which a calibration curve was generated using an authentic standard composed of equal concentrations of php and pyp in 2-propanol. The fluorescence intensity at λ_{em} = 673 was used to generate a calibration curve (Figure 3).

The fluorescence spectra of fecal samples from feedlot cattle, dairy cows, and pigs are presented in Figures 4−6. The feedlot cattle and dairy cow samples give intense peak maxima characteristic of php and pyp. Similarly, the spectra of goats, rabbits, and guinea pigs also produced intense peak maxima at 673 nm (spectral graphs not shown). In contrast, the fluorescence spectra of pigs produced two peak maxima at ∼500 and 673 nm. The broad 500 nm peak, ranging from 450 to 550 nm, results from other unknown fluorescence compounds and is of greater intensity than the 673 nm. It is important to note that this broad 500 nm peak is also produced in the other species of animals, but is of negligible fluorescence intensity in comparison to the peak at 673 nm. The spectra of rat and chicken samples also produced peak maxima at 673 nm, but of much lesser intensity in comparison to the feedlot cattle,

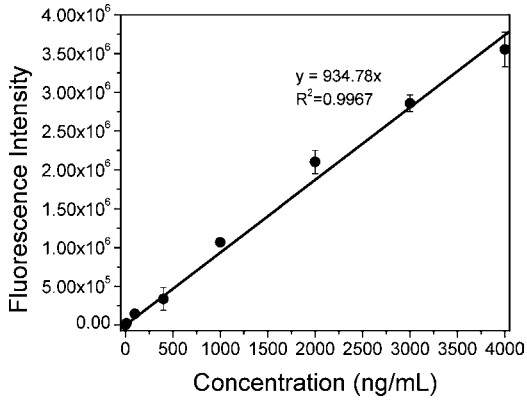

Figure 3. Fluorescence calibration curve of pyp/php (1:1) in 2-propanol (λ_{ex} = 420 nm). The peak maximum of 673 nm was used for calibration.

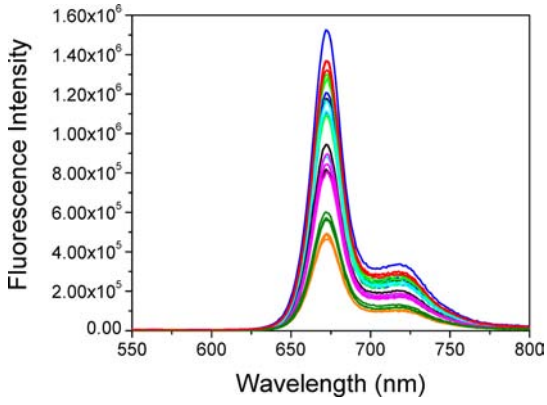

Figure 4. Fluorescence spectra of several samples of feedlot cattle feces in 2-propanol (λ_{ex} = 420 nm).

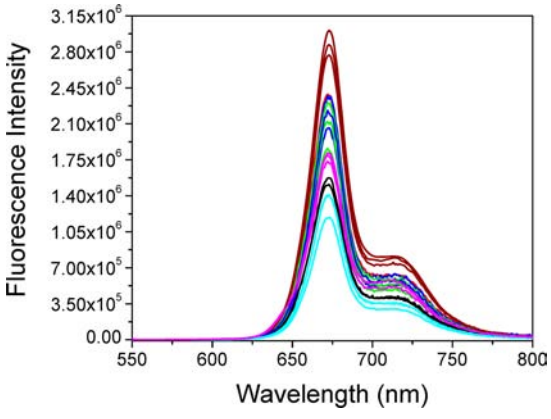

Figure 5. Fluorescence spectra of several samples of dairy cow feces in 2-propanol (λ_{ex} = 420 nm).

dairy cows, guinea pigs, and goats. The average concentrations of pyp/php present in all animal feces are presented in Table 2.

Concentrations were, as anticipated, diet dependent, and low-chlorophyll-containing diets produced correspondingly low levels of php and pyp in feces. The diets of feedlot cattle, dairy cows, guinea pigs, goats, and rabbits had significant quantities of chlorophyll as a result of the feed ingredients, namely, alfalfa meal, alfalfa/grass hay, and fresh pasture (Table 1). The high-level chlorophyll-fed animals possessed greater variability in fecal levels of pyp/php, indicating animal-to-animal variation. In

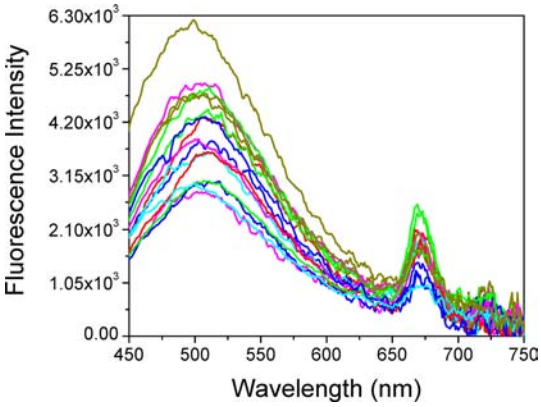

Figure 6. Fluorescence spectra of several samples of pig feces in 2-propanol (λ_{ex} = 420 nm).

Table 2. Average Concentrations of pyp/php in Feces of Several Species of Animals

species	mean fluorescence of max intensity (λ_{em} = 673)	pyp/php (μg/g DM feces)	standard deviation (μg/g)
guinea pig	3.05×10^6	180	±10
goat	2.55×10^6	150	±40
rabbit	2.48×10^6	150	±13
dairy cow	2.01×10^6	120	±29
feedlot cattle	9.73×10^5	60	±20
pig	1.77×10^3	<1	±3
rat	4.14×10^4	<1	±1
chicken	5.49×10^3	<1	±0.12

contrast, pigs had low levels of pyp/php present in their feces. They received a diet consisting primarily of corn and soybean meal, and this diet contained very little green plant material. Rats and chickens also had low levels of pyp/php present in their feces. They consumed commercially available rodent- and chicken-feed diets, which contained minimal levels of chlorophyll-containing feed ingredients.

These results indicate that animals consuming diets with substantial levels of green plant material will excrete feces with concentrations of pheophorbide *a* that greatly exceed (by 10–30-fold) levels demonstrated to be inhibitory to bacterial efflux pumps (0.5 μg/mL).[11,13−15] Although the EPI activity of pyp is not currently known, the natural abundance of php and its properties as an EPI may exert selection pressure upon microbial populations dependent upon efflux pump activity. Inhibitors are known to stimulate synthesis of additional efflux pump capacity as a means of metabolic compensation. This may place an energy burden upon the bacterial cell, and this cost can serve as an environmental factor that selects against antimicrobial resistant phenotypes, which depend upon efflux pumps as a defense mechanism to counter antimicrobial activity. As a result, it is possible that the EPI activity of php may influence the level of antimicrobial resistance expressed in the GI tract environment. In contrast to the antimicrobial resistance displayed in the isolated conditions of pure culture, antimicrobial resistance in the gut may be substantially altered in the presence of plant-derived inhibitors such as php. Given these data, further research is warranted to determine if other EPIs exist and if feed components can modulate the antimicrobial resistance of bacteria in the gut.

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Notes

The authors declare no competing financial interest.

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