Kinetics of the Formation of Zinc Complexes of Chlorophyll Derivatives

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Zinc complexes of chlorophyll derivatives are similar in color to chlorophylls. Unlike the parent compounds, however, they survive heat treatment as observed in the vegetable-processing industry. Little is known about the kinetics of their formation. The reaction of chlorophyll *a* derivatives with zinc(II) ions in 80/20 (v/v) acetone/water was monitored by HPLC. Pyropheophorbide reacted most quickly at 20 °C followed by pheophorbide, methyl pheophorbide, ethyl pheophorbide, pyropheophytin, and pheophytin. Half-life values were 54, 90, 166, 171, 174, and 305 min, respectively. Similar trends were observed at 25, 30, and 35 °C. The E_a was calculated as 21.8 ± 0.5 kcal/mol and was the same for all reactions. Results suggest that steric hindrance from peripheral groups and charge distribution on the tetrapyrrole ring had an effect on the rate of reaction of chlorophyll derivatives with zinc ions.

INTRODUCTION

There has been increasing interest in recent years in the development of food colorants from natural sources (Francis, 1987; Ilker, 1987). Chlorophylls are the most widely distributed plant pigments and might be considered a logical choice for development. Chlorophylls, however, are known to be easily degraded by conditions to which foods are exposed, specifically dilute acids, heat, light, and oxygen (Schanderl et al., 1962; Britton, 1983; von Elbe and LaBorde, 1989).

Zinc complexes of chlorophyll derivatives are similar in color to chlorophylls and have been found in thermally processed green vegetables. As early as 1943, a green substance found to be more stable toward heat compared to chlorophyll was observed in commercially canned okra (Fischbach and Newburger, 1943). Fishbach (1943) later found that the substance was a pigment containing zinc and concluded it was formed by the reaction of zinc with a chlorophyll degradation product. A similar observation was made by Schanderl et al. (1965a,b), who noted that small amounts of green color existed in processed pea puree after storage at elevated temperatures. The pigments responsible for the color were isolated and found to be copper or zinc complexes, mainly of pheophytin *a* with some of pheophytin *b*. A stable green color was also observed by Swirski et al. (1969) in the brine of canned Brussels sprouts. It was suggested that the pigment was a complex of a water-soluble protein containing copper, zinc, and chlorophyll derivatives.

Later, zinc salts were deliberately added to blanch solutions to improve green color in a commercial canning process for peas, beans, and spinach (Segner et al., 1984). The process, known as Veri-Green, produced canned vegetables with brighter color compared to conventionally canned vegetables. The color of Veri-Green vegetables was attributed to the formation of zinc pheophytin *a* and zinc pyropheophytin *a* (von Elbe et al., 1986).

Information about kinetics of the formation of zinc complexes of chlorophyll derivatives is limited. LaBorde and von Elbe (1990) studied zinc complex formation with pyropheophytin *a* in vegetable purees to further understand formation of the metallocomplex during thermal processing. They found that the formation of zinc pyropheophytin *a* in spinach and pea purees was dependent on pigment and zinc ion concentrations as well as pH.

The objectives of this study were (1) to determine reaction rate constants for reaction of the chlorophyll derivatives with zinc(II) ions and (2) to determine the temperature dependence of the reaction.

MATERIALS AND METHODS

Preparation of Chlorophyll Derivatives. Pheophytins were prepared from chlorophylls. The chlorophylls were extracted from 50 g of fresh spinach by blending with 100 mL of acetone. Celite was added, and the mixture was filtered through Whatman No. 1 and No. 42 filter papers in a Büchner funnel. The residue was washed with 50 mL of acetone, and the filtrate was transferred to a separatory funnel. One hundred milliliters of ethyl ether and 100 mL of distilled water were added to produce a biphasic system. The ether layer was separated and acidified with 5 mL of aqueous HCl (1:3 HCl/water v/v) to produce pheophytins. The pheophytins thus produced were washed several times with water, and the final ether solution was dried with granular sodium sulfate, filtered, and evaporated to dryness under vacuum. Pigments were redissolved in 12 mL of acetone, filtered through a 0.2-μm filter, and stored at −15 °C.

Chlorophyllides were prepared from beet leaves according to the method of Holden et al. (1961). Pheophorbides were prepared from chlorophyllides as described above for the preparation of pheophytin from chlorophyll. The method used to prepare ethyl chlorophyllides was similar to a method reported by Holt and Jacobs (1954). Methyl chlorophyllides were similarly prepared except methanol was used in place of ethanol. Methyl or ethyl pheophorbides were prepared from their respective chlorophyllides as previously described for the preparation of pheophytin from chlorophyll.

Pyropheophytins were prepared from fresh spinach, which was blanched at 100 °C for 5 min to minimize chlorophyllase activity. Blanched spinach was packed into no. 303 cans which were filled with water, sealed, and heated in a still-retort at 240 °F for 4 h. The excessive heat treatment was used to hydrolyze the C-10 carbomethoxy group to yield pyropheophytins (Schwartz and von Elbe, 1983). Pyropheophorbides were similarly produced except that spinach was blanched at 70 °C and held for 30 min before retorting. The lower temperature and longer time were used to activate chlorophyllase and allow sufficient time to form appreciable amounts of chlorophyllides (Jones et al., 1963). Pyropheophytins and pyropheophorbides were extracted with acetone, transferred into ether to remove water and water-soluble materials, and stored in 12 mL of acetone as described for pheophytins.

Purification of Chlorophyll *a* Derivatives. Each of the six chlorophyll *a* derivatives was separated from other pigments by C_{18} reversed-phase preparative HPLC. Components of the

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Table I. Preparative HPLC: Conditions for Purification of Chlorophyll *a* Derivatives

pigment	mobile phase	flow rate, mL/min	elution time,[a] min
pheophytin	4:3:1[b]	3	108–130
rechromatographed using a step gradient			
solvent A	65:35:4[c]	3	60[d]
solvent B	acetone	3	49–62
pyropheophytin	4:3:1[b]	3	145–190
rechromatographed using a step gradient			
solvent A	65:35:4[c]	3	60[d]
solvent B	acetone	3	70–80
pheophorbide	63:37[e]	4	110–140
pyropheophorbide	63:37[e]	4	145–190
methyl pheophorbide	65:35:4[c]	3	155–195
ethyl pheophorbide	65:35:4[c]	3	190–240

[a] Fractions collected, 1 tube/min. [b] Ethyl acetate/methanol/water. [c] Acetone/water/ethyl acetate. [d] No fractions collected. [e] Acetone/acetic acid (5% v/v in water).

Table II. Analytical HPLC: Conditions for Separation of Chlorophyll *a* Derivatives and Their Zinc Complexes

pigment	mobile phase	flow rate, mL/min	retention time, min
pheophytin	20:27:5[a]	1.5	10.0
zinc pheophytin			6.0
pyropheophytin	4:3:1[a]	1.5	10.0
zinc pyropheophytin			5.5
pheophorbide	63:37[b]	1.5	7.0
zinc pheophorbide			3.5
pyropheophorbide	63:37[b]	1.5	10.0
zinc pyropheophorbide			4.0
methyl pheophorbide	65:35:4[c]	1.5	6.0
zinc methyl pheophorbide			3.0
ethyl pheophorbide	65:35:4[c]	1.5	10.5
zinc ethyl pheophorbide			4.5

[a] Ethyl acetate/methanol/water. [b] Acetone/acetic acid (5% v/v in water). [c] Acetone/water/ethyl acetate.

HPLC system included a pump (Model 6000A), an injector (Model U6K), a C_{18} column (Bondapak C_{18}/Porasil B, 7.8 mm i.d. × 61 cm each, four columns in series), a detector (Model 440, with a 658-nm filter, set at 0.01 absorbance unit), and a recorder/integrator (Model 740 data module), all of which were manufactured by Waters Chromatography Division, Millipore Corp., Milford, MA. Two-milliliter portions of the 12 mL of concentrated pigment solution were injected onto the preparatory column. Conditions for separations are given in Table I. The eluant was collected in tubes using a fraction collector (ISCO Model 1200 Pup). Fractions containing the appropriate chlorophyll *a* derivative were pooled and transferred to a separatory funnel, and equal volumes of ether and water were added to produce a biphasic system. The ether layer was separated, dried with granular sodium sulfate, filtered, and evaporated under vacuum. Pigments were redissolved in 50 mL of acetone and stored at −15 °C until needed.

Purity of the pigment preparations was confirmed by comparison of light absorption maxima to known literature values (Holt and Jacobs, 1954; Pennington et al., 1964), by known analytical HPLC retention times (Schwartz et al., 1981), and by determination of the absence of extraneous peaks on analytical HPLC chromatograms.

Reaction of Chlorophyll *a* Derivatives with Zinc Ions. Reaction of chlorophyll *a* derivatives with zinc(II) ions was studied at 20, 25, 30, and 35 °C. An excess of zinc(II) ions in the form of the chloride salt was used in all reactions. The reaction system contained the pigment (2.67×10^{-5} M) under study, zinc(II) ions (6.56×10^{-2} M), butylated hydroxyanisole (BHA, 0.3% w/v), and Tween 80 (0.8% w/v), in 80/20 (v/v) acetone/water. BHA was

Table III. Reaction Rate Constants (k), Half-Life Values ($t_{1/2}$), and Energy of Activation Values (E_a) for Reaction of Chlorophyll *a* Derivatives with Zinc(II) Ions at 20, 25, 30, and 35 °C

pigment	temp, °C	slope ± sd[a] (×10^{-3}), min^{-1}	k ± sd,[a] min^{-1} M^{-1}	correl coeff	$t_{1/2}$ ± sd,[a] min	E_a, kcal/mol
pheophytin	20	−0.99 ± 0.02	0.035 ± 0.001	0.998	305 ± 7	22.09
	25	−1.91 ± 0.05	0.067 ± 0.002	0.999	157 ± 3	
	30	−3.37 ± 0.16	0.12 ± 0.01	0.995	89 ± 4	
	35	−6.36 ± 0.16	0.22 ± 0.01	0.998	47 ± 1	
pyropheophytin	20	−1.73 ± 0.01	0.061 ± 0.004	0.998	174 ± 1	22.60
	25	−3.39 ± 0.01	0.119 ± 0.002	0.999	89 ± 1	
	30	−6.41 ± 0.01	0.23 ± 0.01	0.998	47 ± 1	
	35	−11.45 ± 0.01	0.40 ± 0.01	0.999	26 ± 1	
ethyl pheophorbide	20	−1.76 ± 0.01	0.062 ± 0.001	0.998	171 ± 1	21.40
	25	−3.12 ± 0.01	0.110 ± 0.001	0.998	96 ± 1	
	30	−5.65 ± 0.01	0.20 ± 0.01	0.998	53 ± 1	
	35	−10.55 ± 0.01	0.37 ± 0.01	0.998	29 ± 1	
methyl pheophorbide	20	−1.81 ± 0.01	0.064 ± 0.001	0.999	166 ± 1	21.36
	25	−3.28 ± 0.01	0.115 ± 0.001	0.999	92 ± 1	
	30	−6.11 ± 0.01	0.21 ± 0.01	0.998	49 ± 1	
	35	−10.72 ± 0.01	0.38 ± 0.01	0.995	28 ± 1	
pheophorbide	20	−3.36 ± 0.01	0.118 ± 0.001	0.998	90 ± 1	21.52
	25	−5.73 ± 0.01	0.20 ± 0.01	0.999	53 ± 1	
	30	−10.31 ± 0.01	0.36 ± 0.01	0.997	29 ± 1	
	35	−20.47 ± 0.01	0.72 ± 0.02	0.998	15 ± 0.5	
pyropheophorbide	20	−5.57 ± 0.01	0.20 ± 0.01	0.998	54 ± 1	22.06
	25	−10.68 ± 0.01	0.38 ± 0.01	0.998	28 ± 1	
	30	−19.33 ± 0.01	0.69 ± 0.01	0.998	16 ± 0.5	
	35	−35.50 ± 0.01	1.25 ± 0.01	0.998	9 ± 0.5	

[a] sd, standard deviation for duplicate determinations.

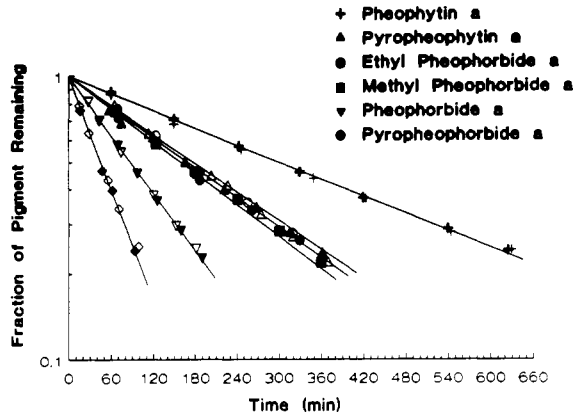

Figure 1. Reaction of chlorophyll *a* derivatives with zinc(II) ions at 20 °C. (Solid and open symbols indicate duplicate trials.)

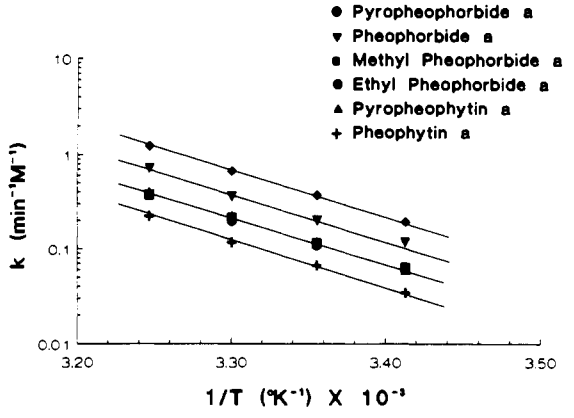

Figure 2. Log of the reaction rate constant (k) vs $1/T$ for reaction of chlorophyll *a* derivatives with zinc(II) ions.

added to minimize oxidation of pigments. Tween 80 was added as a surfactant. Water was boiled before use to remove CO_2.

Reactions were carried out in closed round-bottom flasks in a controlled-temperature water bath (Magni-Whirl, Blue M Electric Co.), protected from light. Separate solutions of pigment (4 mL) and zinc ions (5 mL) were allowed to equilibrate at the desired temperature for 10 min. Two milliliters of zinc ion solution was added to the flask containing the pigment solution, and they were mixed. Twenty microliters was removed at appropriate intervals and injected onto a C_{18} reversed-phase HPLC column. Components of the HPLC system were similar to those used for the purification of chlorophyll *a* derivatives except that the column was a Nova-Pak C_{18} Radial-Pak cartridge, 5 mm i.d. × 10 cm (Waters Chromatography). Mobile phase, flow rate, and retention times for chlorophyll *a* derivatives and their zinc complexes are given in Table II. Peak areas from HPLC chromatograms were compared to appropriate calibration curves to determine concentrations of chlorophyll *a* derivative remaining at each sampling time. A semilog plot of fraction of chlorophyll *a* derivative remaining vs time was used to calculate reaction rate constants and half-life values (Hill, 1977). The temperature dependence of the reaction of pigments with zinc ions was determined by calculating energies of activation from a semilog plot of reaction rate constant vs $1/T$.

RESULTS AND DISCUSSION

Half-life values for the reaction of chlorophyll *a* derivatives with zinc(II) ions were calculated from the slope of each regression line of the log of the fraction of chlorophyll *a* derivative remaining vs time. Linear correlation coefficients indicated straight-line relationships which lead to the assumption that the data fit a pseudo-first-order kinetic model (Hill, 1977). Reaction rate constants were calculated by multiplying the value of the slope of each regression line by 2.303 and dividing the value obtained by the initial zinc ion concentration.

The true order of the reaction was second-order as determined experimentally by varying concentrations of both reactants. The reaction was first-order with respect to the concentration of chlorophyll *a* derivative and first-order with respect to the concentration of zinc(II) ion.

The decrease in concentration of chlorophyll *a* derivative during the course of the reaction was proportional to the increase in concentration of zinc complex formed. Zinc complexes were the only products detected. It was assumed, therefore, that any side reactions were insignificant.

Control samples, in the absence of added zinc ions, did not change in concentration of chlorophyll *a* derivative when exposed to experimental conditions.

Figure 1 illustrates reaction rates of the various chlorophyll *a* derivatives (2.67×10^{-5} M) reacting with zinc(II) ions (6.56×10^{-2} M) at 20 °C. Pyropheophorbide *a* had the fastest rate with a $t_{1/2}$ value of 54 ± 1 min, followed by pheophorbide *a*, methyl pheophorbide *a*, ethyl pheophorbide *a*, pyropheophytin *a*, and pheophytin *a*, which had $t_{1/2}$ values of 90 ± 1, 166 ± 1, 171 ± 1, 174 ± 1, and 305 ± 7 min, respectively. Similar trends were observed for the reaction of chlorophyll *a* derivative with zinc(II) ions at 25, 30, and 35 °C (Table III). The rate at which zinc entered the tetrapyrrole ring decreased as the length of the alkyl chain esterified at the C-7 propionate group increased. These results suggest that steric hindrance has an effect on the rate of reaction of zinc(II) ions with chlorophyll *a* derivatives. This reasoning is supported by Falk (1964), who stated that a displacement mechanism of the S_N2 type is indicated for incorporation of metal ions into porphyrins and a difference in rate between two S_N2 reactions is mainly because of steric factors (Morrison and Boyd, 1973). Schanderl et al. (1962) described a similar result which they ascribed to steric hindrance, in which magnesium ions were displaced by hydrogen ions in the degradation of chlorophyllide *a*, methyl chlorophyllide *a*, ethyl chlorophyllide *a*, and chlorophyll *a* in acid solution. Chlorophyllide *a* had the fastest degradation rate followed by methyl chlorophyllide *a*, ethyl chlorophyllide *a*, and chlorophyll *a*. Berezin and Koifman (1970) reported that, compared to pheophytin *a*, pheophorbide *a* reacted 4 times faster with copper ions in ethanol at 25 °C.

The results of this study further show that the carbomethoxy group at the C-10 carbon on the isocyclic ring had an effect on the rate of reaction of zinc ions with chlorophyll derivatives. Pyropheophorbide *a* and pyropheophytin *a*, which lack a carbomethoxy group at the C-10 carbon, reacted approximately 1.8 times faster with zinc(II) ions compared to pheophorbide *a* and pheophytin *a* at all temperatures. Steric hindrance is one possible explanation for the difference in rate. Another explanation is that the distribution of charge in the aromatic system is affected by the carbomethoxy group which is strongly electron withdrawing (Falk, 1964). In the absence of the carbomethoxy group, the pyrrole nucleus could become slightly more negatively charged, resulting in an increased reaction rate with positively charged zinc ions.

These results suggest that functional groups at the periphery of the tetrapyrrole ring affect the rate of reaction at the nucleus because of steric hindrance, charge distribution, or both.

The temperature dependence of the reaction of chlorophyll *a* derivatives with zinc(II) ions was similar for all derivatives. Figure 2 is a plot of the reaction rate constant (k) vs $1/T$ for the six chlorophyll *a* derivatives. Energy of activation values were similar for all reactions (Table III). The average value for the energy of activation was 21.8 ±

0.5 kcal/mol, which lies within the range 10–30 kcal/mol reported by Lund (1975) for the degradation of food components.

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Registry No. Pyropheophorbide *a*, 24533-72-0; pheophorbide *a*, 15664-29-6; methyl pheophorbide *a*, 5594-30-9; ethyl pheophorbide *a*, 55100-98-6; pyropheophytin *a*, 14409-87-1; pheophytin *a*, 603-17-8; zinc, 7440-66-6.