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Substrate Specificity of Chlorophyllase[1,2]

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ABSTRACT

Apparent Km and V_{max} values were obtained for hydrolysis of methyl and ethyl chlorophyllides *a*, methyl and ethyl pheophorbide *a*, and 9-hydroxymethyl pheophorbide *a* by chlorophyllase from *Ailanthus altissima*. Analysis of substrate specificity data for chlorophyllase indicates that the presence of a 9-keto group and a methyl alcohol group esterified at the 7-position in chlorophyll derivatives results in maximum binding affinity for substrates. Data on maximum reaction rates indicate that the rate-controlling step of hydrolysis occurs after release of the alcohol from the ester. Probable high affinity chlorophyllase inhibitors can be predicted on the basis of these specificity studies.

An improved method for purification of chlorophyllase has been developed.

Quantitative information on the substrate specificity of chlorophyllase is limited. Assay methods suitable for kinetic studies were not available until Klein and Vishniac (5) developed an aqueous detergent system. McFeeters *et al.* (7) did a more complete analysis of the factors which affect chlorophyllase activity. They measured Km and V_{max} values for several chlorophyllase substrates and inhibitors. Kinetic parameters have been determined for some additional chlorophyllase substrates. This information makes possible a more complete picture of the importance of substrate groups to binding and hydrolysis of chlorophyllase substrates.

Structures for Chl derivatives discussed in this paper are given in Figure 1 and Table I.

MATERIALS AND METHODS

Chlorophyllase Preparation. Chlorophyllase was prepared from *Ailanthus altissima* leaves by a rather extensive modification of an earlier procedure (7). An acetone powder was prepared as described in the previous procedure. Chlorophyllase was extracted from the acetone powder with cold, pH 4.60, 0.1 M sodium citrate buffer containing 0.5% Triton X-100 detergent. A ratio of 28 ml of buffer per gram of powder was used for the extraction. The suspension was stirred for 5 min and allowed to stand 20 min. It was centrifuged for 10 min at 19,000*g* in a Sorvall SS-3 centrifuge at room temperature. The supernatant was collected and filtered through loosely packed glass wool to remove floating debris. Twenty-three milliliters of crude extract were obtained from a gram of acetone powder.

Granular ammonium sulfate was added to the crude extract at room temperature (22 C) to give 50% saturation. Twenty minutes after addition of ammonium sulfate, the sample was centrifuged for 10 min at 19,000*g*. A green flotation layer, containing the chlorophyllase activity, was collected from the surface of the liquid. This material was resuspended in pH 7, 5 mM potassium phosphate buffer which contained 0.05 M KCl and 0.2% Triton X-100. Fifteen milliliters of the buffer were used for every 10 g of acetone powder used in the initial extraction. The suspension was centrifuged at 19,000*g* for 10 min and the supernatant was collected.

The chlorophyllase solution was concentrated to one-third of the initial volume in an Amicon Model 402 ultrafiltration cell equipped with an XM-50 membrane. The concentrate was continuously dialyzed in the ultrafiltration cell. A ratio of 5.5 ml of dialysis buffer was put through the cell for each milliliter of enzyme solution. The dialysis buffer was pH 7, 5 mM potassium phosphate which contained 0.05 M KCl. Because the XM-50 filter is not permeable to chlorophyllase, but allows passage of molecules with a mol wt less than 50,000, lower mol wt impurities were removed by this dialysis step.

The chlorophyllase solution obtained after dialysis was clarified by centrifugation and chromatographed on a 2.5 × 15 cm DEAE-cellulose column. Whatman DE-32 microcrystalline cellulose was prepared according to manufacturer's directions and equilibrated with pH 7, 5 mM potassium phosphate buffer containing 0.05 M KCl and 0.2% Triton X-100. A 15-ml sample obtained by extraction of 30 g acetone powder was normally applied to the column. The column was eluted with a 1 liter linear salt gradient. The salt concentration was 0.05 M KCl in the first buffer and 0.4 M KCl in the second buffer.

Fractions from the DEAE-cellulose column with high specific activity were pooled, concentrated by ultrafiltration, and applied to a 1.5 × 100 cm Sephadex G-200 column equilibrated with pH 7, 5 mM potassium phosphate buffer containing 0.05 M KCl and 0.02% Triton X-100. Both ion exchange and gel filtration columns were run at room temperature. After Sephadex G-200 chromatography, chlorophyllase was used for kinetic studies.

Protein determinations were done with either a modification of the biuret method (1) or with the Lowry procedure (6).

Enzyme Assay. The assay procedure for chlorophyllase is described in detail by McFeeters *et al.* (7). However, studies were done with methyl and ethyl alcohol esters of the 7-propionate sidechain. Separation of the esters from the hydrolysis products required a new partitioning solvent. A 1-ml aliquot of reaction mixture was added to a mixture of 3 ml of hexane, 2 ml of acetone, 1 ml of 2-butanone, and 0.2 ml of 0.25 N NaOH, and was shaken. This solvent system

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resulted in nearly complete separation of the ester from the hydrolysis product.

Millimolar absorption coefficients (mM^{-1} cm^{-1}) of 47.2 at 667.5 nm for pheophorbide *a*, 74.9 at 667 nm for chlorophyllide *a*, and 45.0 at 652 nm for 9-hydroxymethyl pheophorbide *a* were used to calculate the amount of product in the reaction mixture. The absorption coefficient for 9-hydroxymethyl pheophorbide *a* in ether was used to calculate the product in the partition mixture. Any difference between the absorption coefficient in the two solvents will result in a proportional error in the apparent V_{max}. However, such an error will not affect the apparent K*m*. Kinetic measurements were made with chlorophyllase which had a specific activity between 50,000 and 100,000 units per milligram of protein.

A unit of chlorophyllase activity is defined as that amount of enzyme which hydrolyzed 1 nmole of pheophytin *a* per hour under standard conditions. Standard conditions are defined as 30 C with a pheophytin *a* concentration of 1×10^{-4} M in a 0.01 M acetate-0.05 M phosphate-0.01 M borate buffer, pH 7.6, containing 0.2% Triton X-100 and KCl to give an ionic strength of 0.3. The standard chlorophyllase concentration was 43.7 units to allow the comparison of newly determined apparent V_{max} values with those from the earlier study (7). Initial reaction rates were obtained by calculation of the slope of the progress curve by a linear regression calculation. Values for apparent K*m* and V_{max} were calculated using the statistical procedure described by Wilkinson (11). All calculations were done on a Wang 600 programable calculator.

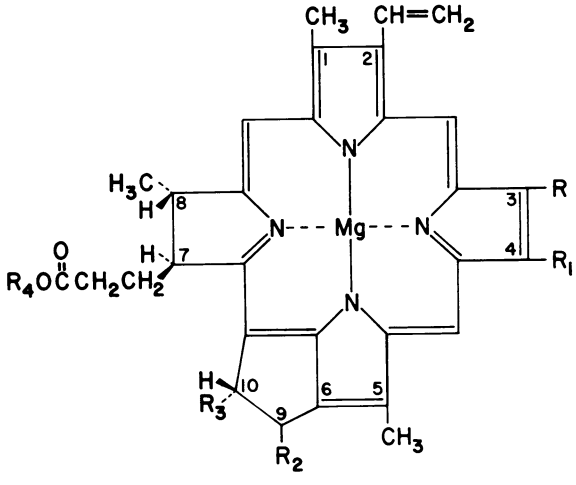

FIG. 1. Ring structure for compounds which are substrates or inhibitors of chlorophyllase.

Preparation of Substrates. Protoporphyrin IX dimethylester was purchased from Sigma Chemical Co. Pheophytin *a* was prepared as described by McFeeters *et al.* (7). Methyl and ethyl chlorophyllide *a* were prepared according to the method of Holt and Jacobs (4). Methyl chlorophyllide *a* was chromatographed by the procedure described by Pennington *et al.* (8). Ethyl pheophorbide *a* was prepared from ethyl chlorophyllide *a* by treatment of an ether solution of ethyl chlorophyllide *a* with 0.5 N HCl.

Methyl pheophorbide *a* was prepared from crystals of impure methyl chlorophyllides *a* and *b* obtained by grinding *Ailanthus altissima* leaves with methanol (4). The crude methyl chlorophyllides were dissolved in a minimum of ethanol and transferred to ether. Ethanol was removed by washing the ether solution with water. The extraction of the ether solution with 18% HCl resulted in immediate conversion of the methyl chlorophyllides to methyl pheophorbides. Methyl pheophorbide *a*, along with some impurities including methyl pheophorbide *b*, was extracted into the aqueous acid phase. The methyl pheophorbide *a* solution was quickly overlayered with ether and diluted with water to transfer the pigment to ether. Pheophorbide *a*, formed during the acid treatment, was extracted with 0.05 N KOH. This was followed by several water washes. Purification of methyl pheophorbide *a* was completed by chromatography on a powdered sugar column using the procedure for purification of ethyl chlorophyllide *a* described by Holt and Jacobs (4). Chromatography by a thin layer procedure (9) showed a single red fluorescent spot.

Methyl pheophorbide *a* was reduced with $NaBH_4$ to give 9-hydroxymethyl pheophorbide *a* (3). Methyl pheophorbide *a* (600 μmoles) was dissolved in 50 ml of benzene. This solution was diluted with 450 ml of methanol. Approximately 0.7 g of $NaBH_4$ was added to the pigment solution and allowed to react for 15 min. The solution was diluted with ether and washed several times with water to remove methanol. The pigments were dried under reduced pressure. Chromatography was done on a 8 × 30 cm powdered sugar column. A developing solvent of 15% chloroform in light petroleum (b.p. 30–60 C) was used. The major band was 9-hydroxymethyl pheophorbide *a*. It was eluted with acetone and rechromatographed on a second powdered sugar column using 0.8%

Table I. *Structures for Compounds Used as Substrates or Inhibitors of Chlorophylase*

See Figure 1 for the ring structure common to these compounds.

Compound	Mg Present[1]	7,8 Reduced[2]	R	R_1	R_2	R_3	R_4
Methyl chlorophyllide *a*	+	+	CH_3	CH_2CH_3	=O	CO_2CH_3	CH_3
Ethyl chlorophyllide *a*	+	+	CH_3	CH_2CH_3	=O	CO_2CH_3	CH_2CH_3
Methyl pheophorbide *a*	−	+	CH_3	CH_2CH_3	=O	CO_2CH_3	CH_3
Ethyl pheophorbide *a*	−	+	CH_3	CH_2CH_3	=O	CO_2CH_3	CH_2CH_3
9 hydroxymethyl pheophorbide *a*	−	+	CH_3	CH_2CH_3	—OH	CO_2CH_3	CH_3
Chlorophyll *a*	+	+	CH_3	CH_2CH_3	=O	CO_2CH_3	phytyl
Chlorophyll *b*	+	+	CHO	CH_2CH_3	=O	CO_2CH_3	phytyl
Pheophytin *a*	−	+	CH_3	CH_2CH_3	=O	CO_2CH_3	phytyl
Pyropheophytin *a*	−	+	CH_3	CH_2CH_3	=O	H	phytyl
Protochlorophyll *a*[3]	+	−	CH_3	CH_2CH_3	=O	CO_2CH_3	phytyl
4-Vinyl protochlorophyll *a*[3]	+	−	CH_3	$CH=CH_2$	=O	CO_2CH_3	phytyl

[1] In Mg-free compounds, the Mg is replaced by 2H.

[2] If 7,8 position is oxidized, 2H are removed and replaced by a double bond.

[3] The stereochemistry of protochlorophyll has not been determined.

Table II. *Purification of Chlorophyllase from Ailanthus altissima*[1]

	Vol	Protein	Activity Units[2]	Recovery	Specific Activity	Purification
	ml	*mg*		%		*fold*
Crude extract	770	3580	2,580,000	100	726	1.0
Resuspended centrifuged flotation layer	43	980	2,590,000	100	2640	3.6
Dialyzed extract (XM-50 ultrafiltration)	40	600	1,860,000	72	3100	4.3
DE-32 chromatography				35[3]	15–25,000	20–35
Sephadex G-200 chromatography				90[3]	100–160,000	140–220

[1] Initial sample was 32 g of *Ailanthus* acetone powder.
[2] One unit of enzyme activity is that amount of enzyme which hydrolyzes 1 nmole of pheophytin *a* per hour.
[3] Based upon the units of activity applied to the column.

Table III. *Millimolar Absorptivity for 9-Hydroxymethyl Pheophorbide a in Ether*

Wavelength	Absorption Coefficient
nm	$mM^{-1}\ cm^{-1}$
395	129
499	13.3
598	3.8
652	45.0

Table IV. *Kinetic Constants for Chlorophyllase Substrates and Inhibitors*[1]

Compound	Km	V_{max}	K_I
	M × 10^5	*moles/l·hr* × 10^5	M × 10^5
Methyl chlorophyllide *a*	11.5 ± 1.4	12.3 ± 0.6	
Ethyl chlorophyllide *a*	7.4 ± 1.1	4.7 ± 0.3	
Methyl pheophorbide *a*	.37 ± .08	5.1 ± 0.2	
Ethyl pheophorbide *a*	1.0 ± 0.2	3.9 ± 0.2	
9-Hydroxymethyl pheophorbide *a*	68 ± 15	7.0 ± 1.2	
Chlorophyll *a*	10.3	5.7	
Chlorophyll *b*	13.4	4.3	
Pheophytin *a*	2.7	5.3	
Pyropheophytin *a*	2.9	0.13	8.5
Protochlorophyll *a*			2.4
4-Vinyl protochlorophyll *a*			3.7

[1] Data for compounds with phytyl esters were obtained in a previous study (7).

isopropanol in light petroleum (b.p. 30–60 C) as the solvent. A single band was obtained. The pigment was eluted with acetone, dried and dissolved in ether. The ether solution was diluted with light petroleum until crystallization of the 9-hydroxymethyl pheophorbide *a* occurred. The crystals were collected and dried.

The spectrum matched that reported by Holt (3) both with regard to absorption maxima and peak ratios. Absorption coefficients for 9-hydroxymethyl pheophorbide *a* were obtained by dissolving a weighed amount of crystals in ether and measuring the absorbance.

RESULTS

Chlorophyllase purification has been significantly improved over that obtained by McFeeters *et al.* (7). However, the chlorophyllase specific activity was not constant across the activity peak obtained by Sephadex G-200 chromatography. This showed that considerable impurities are still present in the chlorophyllase preparation. Repeated chromatography on Sephadex G-200 did not improve the specific activity of the preparation. Purification data are shown in Table II.

Absorption coefficients for 9-hydroxymethyl pheophorbide *a* were obtained so the compound could be measured spectrophotometrically. Absorption coefficients at the absorption maxima in ether are presented in Table III.

Measurements of the hydrolysis of methyl or ethyl esters of chlorophyll derivatives have been slow, laborious, and unsuited for kinetic studies. Quantitative separation of the ester from the hydrolysis product required quantitative chromatography of a reaction mixture and estimation of the amount of pigment in a chromatogram spot. The time and effort required precluded routine analysis of initial rates which are required for a proper kinetic analysis.

For these experiments, a solvent extraction procedure was developed which allows a rapid, quantitative separation of hydrolysis products from the corresponding methyl or ethyl esters. The procedure resulted in only 2% transfer of ethyl chlorophyllide *a* and less than 1% transfer of methyl chlorophyllide *a*, methyl pheophorbide *a*, and 9-hydroxymethyl pheophorbide *a* to the aqueous phase. Transfer of less than 1% pheophorbide *a* to the hydrophobic hexane phase occurred, provided sufficient NaOH was added to ionize the carboxyl group. Because transfer of substrates to the aqueous phase was constant over the time period of the reaction, this transfer did not affect the apparent reaction rate. Transfer of product to the hexane phase would decrease the apparent rate of the reaction slightly.

Protoporphyrin IX dimethylester was tested both as a substrate and an inhibitor of chlorophyllase. No detectable hydrolysis was observed with 2.2×10^{-5} M protoporphyrin IX. In addition, no inhibition of phenophytin *a* hydrolysis was observed with a 3.3×10^{-5} M pheophytin *a* concentration and 2.2×10^{-5} M protoporphyrin IX dimethylester. Insolubility of protoporphyrin IX dimethylester limited the concentration which could be used.

Apparent Km and V_{max} values were calculated for methyl and ethyl chlorophyllide *a*, methyl and ethyl pheophorbide *a* and 9-hydroxymethyl pheophorbide *a*. These values with the calculated standard deviations are presented in Table IV. Also included in Table IV are the kinetic parameters for compounds studied earlier (7). The reactions were run under the standard conditions defined above. In all cases the reaction rates were normalized to the standard chlorophyllase concentration, so the apparent V_{max} values for all compounds are comparable.

DISCUSSION

The best chlorophyllase fractions obtained with the present purification procedure had a specific activity of 160,000 units/

mg of protein, compared to a specific activity of about 20,000 units/mg of protein reported earlier (7). Both repeated gel filtration and cation exchange chromatography failed to improve the specific activity of the enzyme. It seems that high resolution protein purification techniques will be required to complete purification of the *Ailanthus* enzyme.

The large decrease in the Michaelis constant observed for methyl pheophorbide *a*, compared to derivatives with ethyl or phytyl groups at the seven position, indicates that methyl derivatives will be important in future studies of the chlorophyllase. The partition procedure developed for these studies will be very helpful for further investigations with short chain ester derivatives.

It is possible to construct a reasonably well-integrated picture of the effect of substrate groups on chlorophyllase catalysis from the data which are now available. Refer to Tables I and IV for the structures of compounds considered in the following discussion.

The large standard deviations for the apparent K*m* and apparent V_{max} for 9-hydroxy methyl pheophorbide *a* are a result of the very low binding affinity of the enzyme for this compound. It was not possible to suspend sufficient substrate in the buffer to saturate the enzyme. The highest substrate concentration used was 62×10^{-5} M. This compares with an apparent K*m* of 68×10^{-5} M. In addition, an absorption coefficient for 9-hydroxymethyl pheophorbide *a* in ether was used to calculate the amount of 9-hydroxy pheophorbide *a* in the lower phase of the partition mixture. This may result in up to a 10% additional error in apparent V_{max}. However, such a systematic error will not affect the apparent K*m*.

The standard deviation of the apparent K*m* for methyl pheophorbide *a* is relatively large because the lowest substrate concentration for which a rate could be measured was only slightly below the apparent K*m*.

Despite problems with measurement of kinetic parameters at the extremes of substrate concentration, the data demonstrate some significant features of substrate interactions with chlorophyllase. The suggestion by Seiler (10) that the 9-keto group may be important to substrate binding is clearly correct. There is nearly a 200-fold increase in apparent K*m* for 9-hydroxymethyl pheophorbide *a* compared to methyl pheophorbide *a*. The fact that protoporphyrin IX dimethylester did not inhibit chlorophyllase activity at the concentration used indicates that a compound without ring V does not bind well to the enzyme. In view of the importance of the 9-keto group on binding, this is an expected result. The limited solubility of protoporphyrin IX dimethylester in 60:40 ether-butanone did not allow it to be used at a sufficiently high concentration to show that no binding occurs.

Many hydrolytic enzymes transform ester substrates by the general mechanism shown in equation 1:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} \begin{matrix} EP_2 \\ + \\ P_1 \end{matrix} \xrightarrow{k_3} E + P_2 \qquad (1)$$

For ester hydrolysis P_1 is an alcohol and P_2 is the acid portion of the ester. If k_2 controls the reaction rate, variation of the alcohol should cause changes in the apparent V_{max}. However, if k_3 is rate-limiting, the alcohol should have no effect on apparent V_{max}, because EP_2 is identical for a series of derivatives which differ only with respect to the alcoholic substituent. Since pheophytin *a*, ethyl pheophorbide *a*, methyl pheophorbide *a*, Chl *a*, and ethyl chlorophyllide *a* all have an apparent V_{max} from 3.9 to 5.7×10^5 moles/l·hr, this is evidence that for chlorophyllase k_3 is rate limiting. It also shows that the absence of the magnesium ion has little or no effect on apparent V_{max}. Methyl pheophorbide *a* is an exception because it has an apparent V_{max} over twice as large as the other compounds. There is no adequate explanation for this difference at present.

The small differences in apparent K*m* values among Chl *a*, ethyl chlorophyllide *a*, and methyl chlorophyllide *a* indicate that for this group of magnesium-containing compounds the alcohol substituent has only minor effects on substrate binding. However, the apparent K*m* values for pheophytin *a*, ethyl pheophorbide *a* and methyl pheophorbide *a* decrease with decreasing size of the alcohol substituent. This shows that, when the magnesium ion is removed from the tetrapyrrole ring, decreasing the size of the alcohol group improves substrate binding. These data also show that the major effect of magnesium ion removal is to decrease the apparent K*m*. The ratio of the apparent K*m* for the magnesium-containing ester relative to the apparent K*m* of the magnesium-free ester is 3.8 for the phytyl esters, 7.4 for ethyl esters, and 31 for methyl esters.

Only minor effects on substrate binding result from removal of the C_{10} carbomethoxy group (compare apparent K*m* for pheophytin *a* and pyropheophytin *a*), substitution of a formyl group for a methyl group at position 3 (compare Chl *a* and Chl *b*) and oxidation of the 4-ethyl group to a vinyl group (compare protochlorophyll *a* and 4-vinyl protochlorophyll *a*). Oxidation of 7,8 position (compare Chl *a* and protochlorophyll *a*) causes about the same decrease in apparent K*m* as removal of magnesium ion from the tetrapyrrole.

In summary, when the metal ion is removed, the nature of the alcohol esterified at position 7 has an important effect on substrate binding. In addition, the 9-keto group is necessary for strong substrate binding.

The inability of chlorophyllase to hydrolyze protochlorophyll derivatives (2) shows that substrate transformation is completely inhibited by oxidation of the 7,8 positions. The removal of the C_{10}-carbomethoxy group from pyropheophytin *a* results in a large decrease in apparent V_{max} relative to pheophytin *a*. Although the 7,8 hydrogens and carboxymethyl group have relatively small effects on binding, they are important for proper orientation of the ester bond which is hydrolyzed by chlorophyllase.

The substrate specificity data that are available for chlorophyllase show that the enzyme has very specific substrate requirements. This has significant implications for studies directed toward defining the role of this enzyme in chlorophyll metabolism. A comparison of the substrate specificity of chlorophyllase from evolutionary distant plants would indicate the degree of enzyme divergence. If chlorophyllase is an essential enzyme in Chl synthesis or degradation, minimal divergence and very similar substrate specifities would be expected.

An obvious implication from these data is that methyl protopheophorbide and methyl pyropheophorbide *a* derivatives can be expected to be good competitive inhibitors for chlorophyllase. The availability of such inhibitors may be useful for investigations of Chl metabolism.

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