Purification and Properties of Chlorophyllase from
*Ailanthus altissima* (Tree-of-Heaven)\(^1,2\)

Received for publication November 16, 1970

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ABSTRACT

Chlorophyllase from *Ailanthus altissima* leaves has been purified 63-fold by a combination of heat treatment, ultracentrifugation, gel filtration, and chromatography on diethylaminoethyl cellulose. While the enzyme is inhibited to some degree by Triton X-100, a modification of the assay procedure of Klein and Vishniac has been shown to be far superior to the use of aqueous acetone systems. The enzyme was found to have a pH optimum on pheophytin a of 4.5. Chlorophylls a and b, pheophytins a and b, and pyropheophytin a were hydrolyzed by the enzyme while protochlorophyll a and 4-vinyl protochlorophyll a were not hydrolyzed but were competitive inhibitors. \(^*\)p-Nitrophenyl acetate was not hydrolyzed. The enzyme does not appear to contain an essential sulfhydryl group since sodium tetrathionate and \(^*\)p-chloromercuribenzoate did not affect its activity.

Since the discovery of chlorophyllase (chlorophyll chlorophyllidohydrolase, EC 3.1.1.14) by Willsätter and Stoll in 1913 (36), progress in our understanding of both the physiological and of the physical, chemical, and catalytic properties of the enzyme has been quite limited. There is biological evidence consistent with both synthetic (5, 7, 14, 15, 26, 30, 33, 34) and degradative (20, 24, 27, 38) functions for chlorophyllase, but the data are not conclusive for either or both of these possibilities. Acquisition of definitive experimental data for this enzyme has been hindered because both the enzyme and its known substrates are insoluble in aqueous buffers. In the past decade, procedures have been developed in several laboratories (1, 5, 7, 13, 18, 22, 30) for solubilizing chlorophyllase from a number of plant tissues. Solubilization of substrate has been accomplished in nearly all cases by using a buffer containing from 40 to 80% acetone. Interpretation of enzyme kinetics for reactions done under these conditions is difficult. However, a more immediate problem is that substrates are only partially soluble even at high acetone concentrations (1, 3). This makes reproducibility of results very difficult and means that quantitative assays of enzymatic activity are subject to large error. Therefore, most of the quantitative data for chlorophyllase in the literature and interpretations based upon these data are of doubtful validity.

More information about the physical, chemical, and catalytic properties of chlorophyllase is needed before experiments to show the physiological function of the enzyme is likely to be successful. As a beginning to this process, we have partially purified chlorophyllase from *Ailanthus altissima* leaves and studied the effect of detergents, acetone concentration, pH, and ionic strength on the activity of the enzyme. Data on the effect of pH and acetone concentration on stability of the enzyme are also given. Kinetic parameters for some substrates and inhibitors of chlorophyllase have been obtained.

MATERIALS AND METHODS

Chlorophyllase was prepared from young leaves of wild *Ailanthus altissima* trees. Cellex-D (DEAE-\(^*\)cellulose) from Bio-Rad Laboratories was prepared for chromatography according to the procedure of Peterson and Sober (25). Sephadex G-100 was from Pharmacia Fine Chemicals, Incorporated. Crystallized bovine serum albumin was from Armour Pharmaceutical Company. Triton X-100 and alcohol dehydrogenase were obtained from the Sigma Chemical Company. Sprockles concofiers sugar was used for chromatography of plant pigments. All chemicals used were reagent grade. Deionized water prepared by passage through a Barnstead Bantam De-mineralizer was used.

**Preparation of Pigments.** Chlorophylls a and \(b\) were prepared by powdered sugar column chromatography of spinach extracts by the procedure of Strain and Svec (32) with the modification that rechromatography was done with 3% acetone in petroleum ether (30–60 C) for chlorophyll a and with 5 to 6% acetone for chlorophyll b. This gave better separation of chlorophyll from any contaminating pheophytin than did 0.5% \(n\)-propyl alcohol in petroleum ether.

Pheophytin a was prepared from once chromatographed chlorophyll a by adding 5 ml of a 22% HCl solution to 100 ml of an ether solution of chlorophyll a. Conversion was faster if some acetone were added to aid in solubilization of the aqueous HCl in ether. After conversion was completed, petroleum ether was added to give a final ether concentration of 10%. The solution was washed five times with an equal volume of water to remove HCl and acetone. The pheophytin was chromatographed on a powdered sugar column with 3% acetone in petroleum ether as the eluting solvent.

Pheophytin \(b\) was prepared from once chromatographed

\(^1\) This investigation was supported in part by the National Institutes of Health (TO1UI-01053 and AM-11665).

\(^*\) Taken from the dissertation submitted by Roger F. McFeeters to the Graduate Division, University of California, Davis, in partial satisfaction of the requirements for the Ph.D. degree.

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\* Abbreviation: DEAE: diethylaminoethyl.
chlorophyll b by the same procedure as described for pheophytin a except 5 to 6% acetone in petroleum ether was used for the elution solvent.

Pyropheophytin a was prepared from pheophytin a by the procedure of Pennington et al. (23).

Protoporphyrin a and 4-vinyl protoporphyrin a were prepared by a modification of the procedure of Houssier and Sauer (17). The hulled seeds (550 g) were washed twice with 1-liter portions of water, drained, and then extracted in four portions with a total of 1.5 liters of acetone. The seeds were almost completely decolorized. The extract was filtered through glass wool into a separatory funnel. Ether was added, and the pigments were transferred to the ether phase by addition of saturated NaCl solution. After washing 5 to 6 times with water to remove acetone completely, the ether solution was concentrated to 30 ml with a flash evaporator. A water phase separated and was discarded. To complete drying, anhydrous Na2SO4 was added. The pigment was diluted with trimethyl pentane to give a final ether concentration of 10%. This solution was added to an 8-cm diameter column packed with 3 pounds of powdered sugar with a 1-cm layer of anhydrous Na2SO4 on top. The column was developed with a 0.2% n-propyl alcohol in trimethyl pentane.

Protoporphyrin a moved in front of 4-vinyl protoporphyrin a, but there was some overlapping of the bands. The two bands were removed separately, eluted with acetone and rechromatographed on powdered sugar columns. In each case the columns were first eluted with enough 0.25% n-propyl alcohol in trimethyl pentane to move the band from the top of the column. This was followed by pure trimethyl pentane. The pigment bands were again removed and eluted with acetone. Approximately 15 μmoles of protoporphyrin a and 40 μmoles of 4-vinyl protoporphyrin a were obtained. The pigments were characterized by their chromatographic behavior and the positions and ratios of the peaks of the visible spectra.

Ethyl chlorophyllide a was prepared from fresh Ailanthus altissima leaves according to the method of Holt and Jacobs (16).

Assay of Chorophyllase Activity. The assay procedure used was based on the method developed by Klein and Vishniac (18). This method has significant advantages over assays using high acetone concentration to solubilize the substrate. Therefore, it will be discussed in some detail and possible difficulties will be pointed out.

Substrates were added to the reaction as ether solutions. Therefore, it is convenient to measure the concentration of substrate spectrophotometrically after dilution of the ether solution to give a reasonable absorbance. Extinction coefficients in ether for all pigments used in this study are from Smith and Benetiz (31). The molar extinction coefficients for pyropheophytin a and 4-vinyl protoporphyrin a were assumed to be equal to the coefficients for pheophytin a and protoporphyrin a, respectively.

When solutions of pheophytin a, and presumably other chlorophyll derivatives, were kept at room temperature in ether solution for long periods of time, changes occurred in either the solvent or substrate which partially inhibited the enzymatic reaction and gave erratic activity progress curves. No significant change in the absorbance spectrum of pheophytin a was observed. The nature of the change is not understood, but it is accompanied by development of a sharp, unpleasant odor in the solution. The problem can be avoided by keeping solutions refrigerated when not in use and by comparing them with a fresh solution in the enzymic reaction every 4 to 6 weeks.

Klein and Vishniac (18) used an 0.08 m phosphate buffer, pH 7.5, containing 0.2% Triton X-100. This buffer was used for activity determinations during purification. However, for activity measurements in later experiments a 0.01 m acetate-0.05 m phosphate-0.01 m borate buffer, pH 7.6, with KCl added to make the ionic strength 0.3 and containing 0.2% by weight Triton X-100 was used. The advantage is that adequate buffering capacity is present over a wide range of pH.

For each reaction, five graduated 15-ml centrifuge tubes and a test tube were prepared. The centrifuge tubes each contained 1.0 ml of aqueous KOH solution and 10 ml of a 60:40 (v/v) hexane-acetone solution. Hexane gives slightly better partitioning of pheophytins from phophorbides than does petroleum ether which was used by Klein and Vishniac (18). The amount of KOH in 1.0 ml was the amount needed to raise the pH of 1.0 ml of the reaction mixture to 8.5. The reason for this is to make certain the free carboxyl group of the product is ionized during partitioning (see below).

Five milliliters of 0.012 m acetate-0.06 m phosphate-0.012 m phosphate buffer containing KCl and 0.24% Triton X-100 was added to the test tube. When the test tube was shaken vigorously, the volume of water plus enzyme was 0.7 ml. This solution was equilibrated at 30 C, then 0.3 ml of substrate solution was suspended in the buffer by shaking vigorously. Three minutes after adding substrate, enzyme solution was added and mixed to start the reaction. In cases where an acetone powder was used as enzyme, a weighed sample was placed in the buffer before substrate was added. Samples (1.0 ml) were taken at 0, 3, 6, 9, and 12 min after start of the reaction. Each sample was added to a centrifuge tube and shaken vigorously for 5 sec. This stopped the reaction since the enzyme was in the aqueous acetone phase with the product while the unreacted substrate was in the hexane phase.

The samples were centrifuged for 2 min at approximately 1600g in a Model CL International Clinical centrifuge. This gave complete separation of the phases and generally gave lower blank readings. The volume of the aqueous acetone phase was recorded (generally 4.0 ml), and the absorbance of this phase was read at 750 nm to measure light scattering and at an absorption maximum of the product formed. The measurements were done with a Cary 14 spectrophotometer equipped with 0.2 absorbance slidewire.

The following millimolar extinction coefficients (mmoles cm⁻¹) in 80% acetone were used to determine extinction coefficients in 50% acetone: phosphorhodide a, 47.2 at 667.5 nm (18); the extinction coefficient of pyropheophorbide a was assumed to be equal to phosphorhodide a; chlorophyllide a, 74.9 at 667 nm based upon Ziegler and Egle's value of 76.9 for chlorophyll a in 80% acetone (37); chlorophyllide b was assumed to be 47.2 at 650 nm based upon Ziegler and Egle's value of 74.6 for chlorophyll b in 80% acetone (37); and phosphorhodide b, 145.1 at 438 nm and 28.8 at 654 nm based upon Vernon's coefficients for phophorythrin b in 80% acetone (35). Extrapolations for extinction coefficients from 80% acetone to 50% acetone were done in the following way. Substrate was hydrolyzed for an extended period of time under the usual conditions. A 1.0-ml sample was partitioned in hexane-acetone as described above and the absorbance measured. Dilution of 2 ml of this sample to 5 ml with acetone was done to give a final acetone concentration of 80%, and absorbance of the diluted sample was measured. The concentration of product was calculated using the extinction coefficients of the substrate in 80% acetone. The extinction coefficients in 50% acetone were then calculated taking into account the dilution fac-
tor. It was assumed that the millimolar extinction coefficients of substrates and products are equal, since removal of phytol has little or no effect on the visible spectrum.

The moles of product formed were calculated and plotted versus time. The rate of product formation was calculated from the initial slope of the line by use of a Wang 700 computer which was programmed to calculate rates from a least squares line through the points.

**Definition of Units and Standard Conditions.** For the purification procedure, a unit of activity was defined as the amount of enzyme needed to hydrolyze 1 \( \mu \)g of phophytin \( a/ \)hr at 30 C in 0.08 m potassium phosphate buffer, pH 7.5, containing 0.2% Triton X-100. A phosphohtin \( a \) concentration of 60 \( \mu \)g/ml was used.

For the kinetic experiments, standard conditions were defined as 30 C with a 0.01 m acetate-0.05 m phosphate-0.01 m borate buffer, pH 7.6, containing 0.2% Triton X-100 (w/v) and the ionic strength adjusted to 0.3 with KCl. The standard chlorophyllase concentration was that amount of enzyme which hydrolyzed 43.7 nanomoles phophytin \( a \) per hr per ml at an initial phosphohtin \( a \) concentration of 1 \( \times \)\( 10^{-4} \) M. This was approximately equal to 3 \( \mu \)g protein/ml of reaction mixture with the chlorophyllase preparations used in this study.

**Protein Determinations.** The biuret method (12) was used with two modifications. For 1 to 10 mg of protein, 4 ml of biuret reagent was mixed with 1 ml of protein solution. Equal volumes (1.5 ml) of biuret reagent and protein solution were mixed when the range of protein was 0.25 to 2.0 mg (6). In cases where protein concentration was very low, the Lowry method was used (21).

**Purification of Chlorophyllase.** An acetone powder was prepared from fresh, young, fully expanded *Ailanthus altissima* leaves collected in early May. A 50-g sample was ground in a 400-ml Sorvall Omnimixer cup in 350 ml of -20 C acetone for 1.5 min at a rohestat setting of 80 to 90. During grinding the Omnimixer cup was immersed in an ice-salt bath at -8 C. After grinding, the preparation was placed in a Buchner funnel and the acetone removed by suction. Two batches were combined, mixed with 200 ml of cold acetone, regrind, and dried again. This was repeated a second time. The powder was then placed in a vacuum desiccator overnight to complete drying. A yield of 1 g of dried powder for every 5 g of fresh leaves was obtained.

Chlorophyllase was solubilized by extracting the *Ailanthus* acetone powder with cold 0.08 m phosphate buffer, pH 7.5, containing 0.5% by weight Triton X-100. The buffer (30 ml/gram of powder) was added to the powder with constant stirring to prevent formation of lumps. After 5 min of stirring, the suspension was centrifuged for 10 min at 13,000g in a Sorvall RC-2B refrigerated centrifuge. The supernatant liquid contained the solubilized enzyme.

The solubilized enzyme was heated in an Erlenmeyer flask to 60 C for 5 min and then quickly cooled in a bucket of crushed ice. The solution was again centrifuged at 13,000g for 10 min, and the supernatant liquid was collected.

The heated chlorophyllase extract was concentrated by ultrafiltration to approximately 130 ml and dialyzed in the ultrafiltration cell with three times this volume of a pH 7.5 buffer of proper concentration so that the chlorophyllase was in a solution of 5 \( \times \)\( 10^{-8} \) M phosphate, 0.1 M KCl, and 0.2% Triton X-100 at conclusion of dialysis. After dialysis, the solution was concentrated to 40 ml. For ultrafiltration, an Amicon model 401 ultrafiltration cell, with a capacity of 400 ml, attached to a 2.75-liter fiberglass reservoir was used with an XM-50 membrane with a molecular weight cut-off of 50,000. Chlorophyllase did not pass through this membrane.

In small scale separations of the enzyme a gel filtration step was included at this point (Table 1). A Sephadex G-100 column (3.5 \( \times \) 60 cm) was packed and equilibrated with 0.08 m phosphate buffer, pH 7.5, containing 0.2% by weight Triton X-100. A maximum of 50 ml of enzyme solution was applied to the column at a time. The enzyme eluted near the void volume of the column. The gel filtration step was eliminated in subsequent large scale purifications of the enzyme without significant effect on over-all fold purification.

DEAE-cellulose was prepared by the procedure of Peterson and Sober (25) in a final buffer of pH 7.5 containing 5 \( \times \)\( 10^{-3} \) M phosphate, 0.1 M KCl, and 0.2% Triton X-100. For a 40-ml sample a 2.1- \( \times \) 45-cm column was used. The column was eluted with a 2-liter linear salt gradient with the buffer containing 0.1 M KCl in the first chamber and 0.5 M KCl in the second chamber.

**Surface Tension Measurements.** Apparent surface tension was measured with a Cenco Du Nouy interfacial tensiometer equipped with a platinum ring 6 cm in circumference.

**RESULTS**

**Purification of Chlorophyllase from *Ailanthus altissima*.** Data for the purification of chlorophyllase are given in Table I. In addition to the Triton X-100 method, solubilization of the enzyme was attempted with the procedure of Klein and Vishniac (18), and with 0.08 m phosphate buffer, pH 7.6, containing 0.5 M NaCl. Both of these methods failed to give significant amounts of soluble enzyme.

Ammonium sulfate and acetone precipitations both resulted in large losses of total activity and precipitates which were difficult to resuspend. Therefore, a heat step was used. It did not precipitate the enzyme and did not destroy too much activity.

When Sephadex G-100 chromatography was performed on the heated extract, about a 5-fold purification was obtained. However, after ultrafiltration, less than a 2-fold increase of specific activity was observed. As a result, gel filtration was eliminated from the procedure.

Ultrafiltration of the heated extract resulted in a 3-fold purification of chlorophyllase with a very small loss in ac-

| Table I. Purification of Chlorophyllase from *Ailanthus* Leaves |
|---|---|---|---|---|
| **Sample** | **Volume** | **Protein** | **Units Activity** \( \times 10^{4} \) | **Recovery of Activity** | **Specific Activity** | **Purification** |
| Crude extract | 280 | 3080 | 952 | 100 | 309 | 1.0 |
| Heated extract | 280 | 1694 | 790 | 83 | 466 | 1.5 |
| Ultrafiltration contents | 40 | 584 | 750 | 79 | 1,318 | 4.2 |
| Ultrafiltration effluent (initial) | 125 | 213 | 3 | 0.3 | 14 | |
| Sephadex G-100 | 15 | 314 | 698 | 73 | 2,220 | 7.2 |
| DEAE applied | 15 | 171 | 435 | 25 | 2,550 | |
| DEAE recovered | 7.1 | 139 | 25 | 19,600 | 63 | |

1 One unit enzyme activity is equal to the amount of enzyme which hydrolyzes 1 \( \mu \)g of phophytin \( a \) per hour.
2 Extracted from 12 g (10 \( u \) units enzyme activity/g) of acetone powder of *Ailanthus altissima* leaves.
3 This step was eliminated in large scale purification of the enzyme without significant effect on over-all fold purification.
4 Expected recovery if all of the enzyme from the Sephadex column had been added to the DEAE-cellulose column.
activity. In addition, concentration and dialysis could be performed in the same step. The concentration step is important for the purification of larger quantities of enzyme because it reduces the volume of solution which must be added to the DEAE-cellulose column.

Figure 1 gives the elution profile from DEAE-cellulose for the sample described in Table I. The chlorophyllase peak is eluted from the column at a KCl concentration of 0.13 M. No distinct protein peak was observed to coincide with the chlorophyllase peak. Chlorophyllase, after DEAE-cellulose chromatography, was used in the kinetic experiments. For preparation of larger amounts of enzyme, some degree of purification was sacrificed to obtain larger yields, so the specific activity of the enzyme was about 11,000 units/mg protein instead of the 19,600 units/mg obtained in the best preparation. The enzyme preparation was stable for over a year when stored at 4°C in 5 × 10^{-4} M phosphate buffer, pH 7.5, containing 0.1 M KCl, and 0.2% Triton X-100.

Evidence that Chlorophyllase Activity was Observed. Grob and Seiler (13) pointed out that when chlorophyllase reactions are performed in air, care must be taken to be sure that enzymatic oxidation of the substrate is not mistaken for enzymatic hydrolysis by chlorophyllase. Hydrolysis was confirmed by two pieces of evidence. First, the product of pheophytin a hydrolysis gave a single spot on thin layer chromatography with the same Rf as a known pheophorbide a sample. Second, hydrolysis of ethyl chlorophyllide a was measured by following the formation of DPNH at 340 nm when the ethanol produced was oxidized with alcohol dehydrogenase. Technical difficulties made it impossible to use this assay for quantitative measurements. However, since the only source of ethanol was from hydrolysis of ethyl chlorophyllide a, occurrence of the reaction is strong evidence for hydrolysis rather than oxidation.

Fig. 3. Inhibition of pheophytin a hydrolysis by Triton X-100. Conditions were as in Figure 2 except for variable Triton X-100 concentration. Chlorophyllase concentration was the amount needed to hydrolyze 43.7 nmoles per ml per hr of pheophytin a at initial concentration of 1 \( \times 10^{-4} \) M. The velocity, \( v \), is expressed as nmoles per ml per hr of pheophytin a hydrolyzed. O: 0.78 \( \times 10^{-4} \) M pheophytin a; x: 2.0 \( \times 10^{-4} \) M pheophytin a.

A suspension of 6.8 \( \times 10^{-4} \) M pheophytin b was made under standard conditions and then diluted with buffer to 0.57 \( \times 10^{-4} \) M. Immediately after dilution a large portion of the pheophytin b was in the 674 nm form. Enzymatic reactions were run immediately after dilution and 90 min later when all of the pigment had converted to the 657 nm form. The rate of hydrolysis was 1.6 times faster in the 90-min sample than in the zero time sample.

It was found that when the Triton X-100 concentration was increased, the proportion of short wavelength, 657 nm, form of pheophytin b in the buffer increased. Therefore, in a second experiment, reactions were done with 2.3 \( \times 10^{-4} \) M pheophytin b as substrate in 0.2% and 0.6% Triton X-100. The observed rate in 0.2% Triton X-100 was 14 nmoles per hr per ml. After correction for Triton X-100 inhibition, the rate in 0.6% Triton X-100 was 28.4 nmoles per hr per ml. The amount of 657 nm form of pheophytin b in a 2.3 \( \times 10^{-4} \) M solution was found to be 0.76 \( \times 10^{-4} \) M in 0.2% Triton X-100 and 1.4 \( \times 10^{-4} \) M in 0.6% Triton X-100. Calculations of the expected rate in 0.6% Triton X-100 were made for a first order reaction and for Michaelis-Menten kinetics with the \( K_m \) and \( V_{max} \) values given in Table IV. The calculated rates were 26.2 nmoles per hr per ml for a first order rate and 14.6 nmoles per hr per ml for Michaelis-Menten kinetics. The corrected observed rate is equal to the first order calculated rate. Since the rate is proportional to the concentration of 657 nm form of pheophytin b in solution, it can be concluded that the 674 nm pheophytin b is not hydrolyzed by chlorophyllase. An examination of (S)/v versus (S) curves for pheophytin a indicated that the long wavelength, 693 nm, form of pheophytin a probably was not hydrolyzed by chlorophyllase.

Chlorophylls a and b, pheophytin a, protochlorophyll a, and 4-vinyl protochlorophyll a gave only a single red peak when suspended under the same conditions as the pheophytns.

Triton X-100 micelles have been estimated to have a molecular weight of 90,000 by light scattering (8, 19) and 63,000 by ultracentrifugation studies (10) and to consist of 140 detergent monomers and 6000 water molecules with a radius of 43 Å (19). An attempt was made to separate the two forms of pheophytin a based on the premise that they are located in different size detergent micelles or that one form is not in a micelle. A Sephadex G-100 column (1 \( \times \) 30 cm) was equilibrated with the standard buffer. A 1-ml sample of this buffer containing 1 \( \times 10^{-4} \) M pheophytin a was passed through the column. Both the short and long wavelength forms of pheophytin a were eluted near the void volume. No significant amount of pheophytin a was eluted in later fractions. Therefore, essentially all of the pigment is located in large micelles.

The problems caused by both detergent-enzyme and detergent-substrate interactions led to a search for another detergent to solubilize substrates for chlorophyllase. The following surfactants were tested for their ability to solubilize 9 \( \times 10^{-4} \) M pheophytin a: 0.4% sodium dodecyl sulfate, 0.4% sodium desoxocholate, 2% Tween 80, 2% Tween 20, 2% gum arabic, 1% lubrol, 1% cetyl pyridinium bromide, 1% cetyl trimethylammonium bromide, 0.4% Triton X-102, 0.4% Triton X-165, and 0.4% Triton X-305. Even though pheophytin a was suspended in most cases, none of the surfactants was satisfactory. The pigment formed visible particles in every case, making interpretation of results as uncertain as when the aqueous-acetone system was used.

Effects of Acetone Concentration on Activity of Chlorophyllase. Nearly all studies of chlorophyllase have used an assay in which 40 to 80% acetone was used to solubilize the substrate. Therefore, it is necessary to compare results obtained in a Triton X-100 system and in various acetone concentrations.

Solubility problems and pH shifts of the buffer ions at high acetone concentrations made it necessary to use a 0.02 M formate-0.02 M acetate buffer with KCl added to give an ionic strength of 0.05. Bacarella et al. (2) have shown good agreement between the results obtained in this system and in the aqueous-acetone systems.

Fig. 4. Spectrum of pheophytin a suspended in 0.01 M acetate-0.05 M phosphate-0.01 M borate buffer, pH 7.6, containing 0.2% Triton X-100. A is the spectrum of 2 \( \times 10^{-4} \) M pheophytin a immediately after dilution from a concentration of 1.4 \( \times 10^{-4} \) M; B is the spectrum of the same sample after 90 min at 23 C.
However, with 2 decrease in catalytic Km could be found to increased to 4.2. The activity assays of chlorophyllase were run in the usual way. Chlorophyllase was found to be completely stable at pH 6.2 for 3 min in 70% acetone. After 60 min in 70% acetone, only a 10% decrease in activity occurred. At pH 4.2 the enzyme is much less stable. Only 35% of the initial activity remained after 3 min in 70% acetone. The 60-min sample retained only 6% of the initial activity.

The effect of acetone concentration on the rate of pheophytin a hydrolysis by chlorophyllase was determined at pH 4.2 and 6.2. Results are shown in Figure 6. Reactions were done with 2 × 10⁻⁴ M and 1.4 × 10⁻⁴ M pheophytin a in 0, 15, 30, 50, and 70% acetone. Each reaction was done in duplicate. Approximate Km and Vmax values were calculated at each acetone concentration by use of velocities determined at the the two substrate concentrations. A steady decrease of both Km and Vmax occurred as acetone concentration was increased at each pH except at pH 6.2 in 70% acetone where Vmax increased to nearly the same level as in the absence of acetone. However, Km increased greatly from 2.5 × 10⁻⁴ M in 50% acetone to 3.6 × 10⁻⁴ M in 70% acetone. This resulted in a decrease in catalytic efficiency, Vmax/Km, from 4.6 in 50% acetone to 0.1 in 70% acetone. Data for 70% acetone at pH 4.2 could not be obtained due to enzyme instability.

The effect of acetone concentration on chlorophyllase activity in the presence and absence of Triton X-100 is shown in Table II. It should be emphasized that these are initial rate data.

**Effect of pH on Chlorophyllase Activity.** The pH optimum of chlorophyllase from a number of plants has been reported to be between pH 6 and 8. However, the effects of pH on enzyme instability and on ionization of groups in the active site of the enzyme have not been separated. Also, reactions have been done at a single substrate concentration without reporting its relationship to Km. Therefore, it appeared desirable to study the pH-activity profile in greater detail.

Stability of chlorophyllase as a function of pH was measured using standard conditions except for adjustment to a series of pH values. Enzyme was incubated at each pH, and

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**Table II. Effect of Acetone Concentration on Chlorophyllase Activity**

<table>
<thead>
<tr>
<th>Acetone %</th>
<th>Activity</th>
<th>0.2% Triton X-100</th>
<th>No Triton X-100</th>
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<tbody>
<tr>
<td>0</td>
<td>43.0</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>43.1</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>18.9</td>
<td>3.5</td>
<td></td>
</tr>
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<td>50</td>
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</tr>
<tr>
<td>70</td>
<td>11.4</td>
<td>10.1</td>
<td></td>
</tr>
</tbody>
</table>

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**FIG. 5.** Stability of chlorophyllase in aqueous acetone at pH 4.2 and 6.2. Enzyme was incubated at the proper acetone concentration at 30 C. Activity left was measured at pH 6.2 in 0.02 M formate-0.02 M acetate buffer, V = 0.05, containing 0.2% Triton X-100. O and •: 3- and 60-min incubation at pH 6.2, respectively; ∆ and △: 3- and 60-min incubation at pH 4.2, respectively.

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**FIG. 6.** Effect of acetone on the apparent Km and Vmax of pheophytin a hydrolysis by chlorophyllase. Reactions were done at 30 C in 0.02 M formate-0.02 M acetate buffer, V = 0.05, with 0.2% Triton X-100. The pH was measured at the acetone concentration used. The chlorophyllase added was the amount needed to hydrolyze 43.7 nmoles per ml/hr pheophytin a at 30 C in pH 7.6 buffer with 1 × 10⁻⁴ M substrate. X----X: Vmax at pH 4.2; O--O: Km at pH 6.2; X---X: Km at pH 6.2.
samples were removed at intervals for activity assays. Nearly complete stability was found between pH 3.7 and 9. The enzyme was sufficiently stable for 12 min so that reactions could be done from pH 3.2 to 12 with little difficulty due to instability. Results are shown in Table III.

As a first step in evaluation of the effect of pH on activity of the enzyme, reactions were done at a single pheophytin a concentration of $1 \times 10^{-4}$ M (Fig. 7). Highest activity was obtained at pH 7.4 using 1 $\times 10^{-4}$ M pheophytin a.

Table III. Effect of pH on the Stability of Chlorophyllase

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial Activity Remaining after Incubation</th>
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<tr>
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<tr>
<td>2.27</td>
<td>17.5</td>
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<td>2.75</td>
<td>19.5</td>
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<td>3.18</td>
<td>97</td>
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<td>3.72</td>
<td>104</td>
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<tr>
<td>4.71</td>
<td>99</td>
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<td>5.72</td>
<td>97</td>
</tr>
<tr>
<td>6.74</td>
<td>93</td>
</tr>
<tr>
<td>7.20</td>
<td>89</td>
</tr>
<tr>
<td>9.18</td>
<td>103</td>
</tr>
<tr>
<td>11.1</td>
<td>83</td>
</tr>
<tr>
<td>11.9</td>
<td>61</td>
</tr>
</tbody>
</table>

For activity, the pH was first varied. No product was formed. No KOH was added to the partition mixture in assaying for product formed.

Fig. 8. $K_m$ and $V_{max}$ values for pheophytin a hydrolysis of chlorophyllase as a function of pH. Reaction conditions were as in Figure 7 except variable substrate concentration used for determination of $K_m$ and $V_{max}$. •: $V_{max}$; Δ: $K_m$; ---; theoretical $V_{max}$-pH curve based on $pK_a$ and $pK_b$ of 3.4 and 5.6.

served at pH 8, but only a small decrease in activity occurred in the alkaline region. Below pH 8 the activity decreased rapidly. Since the substrate has no ionizable groups, this decrease appeared to be a result of protonation of a group on the enzyme with a $pK$ near 6.5.

Subsequently, it was found that the $pK$ of 6.5 was an artifact caused by transfer of protonated product to the hexane phase in the assay procedure. This problem was corrected by adding 1 ml of a predetermined concentration of KOH to each hexane-acetone partition mixture during the assay for the amount of product formed. The base added was sufficient to raise the pH of the buffer to 8.5 and completely ionize the product.

The reason for apparent decrease in activity was confirmed by suspending $1 \times 10^{-4}$ M pheophorbide a in buffers of different pH and measuring the retention in the aqueous acetone phase. In Figure 7, the dashed curve is a theoretical dissociation curve with a $pK$ of 6.2. The X’s are experimental points. The good fit of the data and the correlation with the observed decrease in activity at low pH make it clear that transfer of protonated product to the wrong phase is the reason for the apparent decline in activity.

Figure 8 shows the pH profile from pH 3.2 to 7.7 found for pheophytin a when the rate of hydrolysis was measured properly. $K_m$ and $V_{max}$ at each pH were calculated from (S)/v versus (S) plots of data at six pheophytin a concentrations from $0.5 \times 10^{-4}$ M to $1.8 \times 10^{-4}$ M done in duplicate. The slope and intercept of a least squares line through the points were calculated using a Wang 700 computer. It should be noted that the presence of the 693 nm form of pheophytin a at the higher substrate concentrations will tend to lower the values of both $K_m$ and $V_{max}$. However, the relative values at different pH values are not affected to any large degree. Therefore, the interpreta-
Table IV. Kinetic Constants for Chlorophyllase Substrates and Inhibitors

Reactions were done in 0.01 M acetate-0.05 M phosphate-0.01 M borate buffer, pH 7.6, μ = 0.3, containing 0.2% Triton X-100 at 30 C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>K_m ( \times 10^{-4} ) mM</th>
<th>V_max[\text{H}^+] ( \times 10^{-4} ) μmol/min/mg</th>
<th>K_i ( \times 10^{-4} ) mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>10.3</td>
<td>5.7</td>
<td>13.4</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>13.4</td>
<td>4.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Pheophytin a</td>
<td>2.7</td>
<td>5.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Pheophytin b</td>
<td>0.35</td>
<td>0.91</td>
<td>2.9</td>
</tr>
<tr>
<td>Pyropheophytin a</td>
<td>2.9</td>
<td>0.13</td>
<td>8.5</td>
</tr>
<tr>
<td>Protoporphyrin a</td>
<td>2.4</td>
<td>1.1</td>
<td>3.7</td>
</tr>
<tr>
<td>4-Vinyl protoporphrin a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values. It is not possible to say whether K_m is a dissociation constant. Compared to pheophytin a, the V_max[H+] values are nearly the same, but K_m values are three to four times lower for chlorophylls a and b.

By analogy, it might be expected that the K_m and V_max[H+] values for pheophytins a and b would be very similar to each other. However, both constants are very much lower for pheophytin b. The reason for this is believed to be a result of the buffer becoming saturated at very low pheophytin b concentrations with the active 657 nm form of the substrate. The experiment described in the section on the interaction of substrates with Triton X-100 is consistent with the possibility that the true K_m and V_max[H+] are larger than the values observed in these experiments. Therefore, the constants given in Table IV for pheophytin a are thought to represent parameters describing the saturation of the buffer with 657 nm pheophytin b rather than parameters of the enzyme-catalyzed reaction. If a system could be found which prevented the formation of 674 nm pheophytin b, it is expected that the K_m and V_max[H+] observed would be similar to those for pheophytin a.

Pyropheophytin a has the carbamothoxy group at C_6 of pheophytin a replaced by a hydrogen. K_m is about the same as for pheophytin a, but V_max[H+] is only 2% as great. Since K_m of pheophytin a appears to be a dissociation constant and the catalytic step for pyropheophytin a is much slower than for pheophytin a, it is likely that the K_m of pyropheophytin a is also a dissociation constant. A problem with these data is that a K_i of 8.5 \times 10^{-5} M was found when pyropheophytin a was used as an inhibitor of pheophytin a hydrolysis. This intersection of the lines in the 1/v versus (1/p) plot was on the x-axis indicating noncompetitive inhibition. Neither the reason for a K_i larger than K_m or for noncompetitive instead of the expected competitive inhibition is known.

Earlier observations that protoporphyrin III and its derivatives are not hydrolyzed by chlorophyllase have been confirmed for protoporphyrin a and 4-vinyl protoporphyrin a. However, both compounds inhibited the hydrolysis of pheophytin a competitively. Furthermore, if the K_i values are compared to the K_m of pheophytin a, they appear to bind about as well as pheophytin a.

Finally, the effect of 2 \times 10^{-5} M pyrrole as an inhibitor of pheophytin a hydrolysis was determined. It had no effect on the rate of hydrolysis, so it is concluded that, at the concentration used, pyrrole does not bind to the substrate binding site of chlorophyllase.

**DISCUSSION**

A careful evaluation of the effect of acetone on the activity of chlorophyllase showed that activity is much lower in aqueous acetone systems. When pheophytin a was used as substrate, considerable precipitation of substrate occurred at all acetone concentrations up to 70%. This is probably also true for chlorophylls (3). This problem makes assays in aqueous acetone systems unsuitable for kinetic studies, since reproducibility of measurements under these conditions would be very difficult. Therefore, quantitative data on chlorophyllase activity obtained with assays in aqueous acetone media should be regarded with considerable caution. Despite the difficulties involved in working with detergents, we believe the Triton X-100 assay is superior to the aqueous acetone system and should be used in future studies in preference to the old method.

It has been necessary for different workers to use a different solubilization procedure for chlorophyllase from each tissue studied. However, in addition to solubilizing chlorophyllase from *Allantia* acetone powder, extraction with pH 7.5 buffer containing 0.5% Triton X-100 resulted in considerable solu-
bilization of the enzyme from acetone powers of etiolated e seedlings and eey grass. It is possible that this procedure will be more general than other methods devised to date. Comparisons of preparations made in other laboratories are difficul because of the differences in the methods of assay and of reporting results. However, our best preparation has about 10 to 15 times higher specific activity compared to the best enzy prepared by Klein and Vishniac (18). Also, they had so little material remaining after the last step that enzyme with about the same specific activity as our crude extract was used for their kinetic studies.

Inhibition of chlorophyllase activity by Triton X-100 is not well understood at present. Within the range of detergent con centrations studied, surface tension data indicated the mono mer concentration was constant. Therefore, changes in the observed rate must be a result of changes in the detergent micelle concentration. The kinetic data for pheophytin a (Fig. 3), and also for chlorophyll b, do not show simple competitive or noncompetitive inhibition. There are three possible explana tions for the observed decrease in reaction rate. First, the detergent micelle may bind to the enzyme active site and pre vent entry of substrate. Secondly, the micelle could bind to the enzyme at some other site which would allow binding of substrate, but still slow or prevent the rate of reaction. The third possibility is a dilution effect. Sephadex G-100 chromatography showed the substrate to be located in micelles of Triton X-100. As the detergent concentration is increased for a given sub strate concentration, the amount of substrate per micelle would decrease. If the enzyme is then able to hydrolyze fewer mole cules of substrate with each interaction with a micelle, a reduc ed rate would be observed. Further experiments are needed to evaluate the contributions of each of these possible factors to the observed inhibition.

The pH-activity profile observed for Alilanthus chlorophyl lase is quite different from previous results where pH optima of 6 to 8 were observed. There are several possible reasons for this. First, the Alilanthus enzyme may be different from en zymes of other plants in this respect. Secondly, the pH of some prototrophic groups can shift a good deal when the dielectric constant of the medium is decreased. For example, acetic acid has apparent pK values of 4.7, 6.0, and 7.3 in 0, 50% and 80% acetone, respectively. Since a high acetone concentra tion has usually been used in the assays, this may have shifted the pH-activity profile to higher pH. Thirdly, we have found the enzyme to be more unstable at low pH in the presence of acetone (Fig. 5) than it is in aqueous medium. Since reaction times of 30 min or greater have been used in most studies and correction for enzyme instability has not been done, decreased activity at low pH may have been due to enzyme denaturation. Finally, the observation that an apparent decrease of reaction rate at low pH (Fig. 7) is a result of protonation of the product and its transfer to hexane, where it is not measured by the assay method, suggests this may have occurred in earlier ex periments. Holden (15), Klein and Vishniac (18), Böger (5), Ogura and Takamiya (22) and Seiler (28) all used a sol vent partition method in their assay during which this transfer could occur. None of these investigators indicated that the pH was increased before partition. Therefore, it is possible that the observed decrease of activity at low pH was a result of an error in the assay procedure. Only the data of Shimizu and Tamaki (30), who chromatographed the reaction mixture on paper, is free from this criticism. However, the reactions were run for 1 hr in 64% acetone so the possibilities of pK shifts and denaturation of the enzyme are still present.

Several experiments have contributed to a knowledge of the factors which contribute to the ability of chlorophyllase to bind and hydrolyze possible substrates. The lack of general esterase activity on iso-amyl benzoate reported by Seiler and Grob (29) is confirmed with p-nitrophenyl acetate. The inhibition of etiolated e seedling chlorophyllase by p-chloroammonium reported by Klein and Vishniac (18) was not observed for the Alilanthus enzyme. Seiler (28) proposed that the substrate is held in the active site of chlorophyllase by hydrogen bonding of the 7 and 8 hydrogens to the sulfur atoms of a disulfide bond and by a coordination bond between the keto group at C-9 and a metal ion in the enzyme. There are several arguments against this proposal. Sulfur atoms are not likely to form hydrogen bonds. This type of bonding appears more unlikely since Fleming (11) and Brockmann (4) estab lished the absolute configuration of chlorophyll which places the 7 and 8 hydrogens on opposite sides of the plane of the porphyrin ring. The evidence that protochlorophyll a and 4-vinyl protochlorophyll a, which lack these hydrogens, bind to chlorophyllase indicates this type of hydrogen bonding is not necessary for substrate binding. There is no experimental evidence to show whether the suggested C-9 interaction with a metal ion (28) may occur.

LITERATURE CITED


