Activity and Partial Purification of Chlorophyllase in Aqueous Systems*

ATTILA O. KLEIN† AND WOLF VISHNIAC

From the Department of Microbiology, Yale University, New Haven, Connecticut

(Received for publication, March 15, 1961)

Enzymatic hydrolysis of chlorophylls into chlorophyllides and phytol, and transesterification to the corresponding methyl and ethyl esters, was reported by Willstätter and Stoll in 1913 (1). Krossing (2) found such activity localized in the chloroplasts, while more recently Ardaio and Vennesland (3) showed that spinach chloroplasin exhibits chlorophyllase activity. In these and other (4) investigations, however, chlorophyllase had been demonstrated only in crude preparations such as acetone powders and pastes and only in reaction mixtures containing a high proportion of organic solvents. Holden (5) recently succeeded in extracting some sugar beet chlorophyllase with citrate and achieved further purification by acetone precipitation. We have partially purified the enzyme from etiolated rye chloroplasts, and determined some of its properties in an aqueous assay system.

**EXPERIMENTAL PROCEDURE**

*Source of Enzyme*—In agreement with previous reports we found chlorophyllase activity in Ailanthus, parsley, stinging nettle, and dandelion leaves. Barley and oat seedlings grown in the laboratory were very poor sources, but etiolated rye seedlings (Detra-Petkus variety of winter rye) grown for 7 to 8 days proved superior in specific activity to other plants tested and were adopted as starting material. A preparation with good activity was also obtained from Euglena gracilis.

**Assay Procedure**—Three assay procedures were employed in this study, depending on the nature of the substrate and the stage of purification of the enzyme. In the course of screening various sources for chlorophyllase, the method of Weast and Mackinney (6) was employed. In this method, a weighed amount (10 to 20 mg) of leaf acetone powder is freed of chlorophyll and then incubated for various periods (3 to 18 hours) in 66% aqueous acetone containing a known amount of substrate. At the conclusion of the hydrolysis, the suspension is filtered, the residual ether could be removed under reduced pressure, the method may be used even though the enzyme is present in an insoluble form as was the case in the early steps of purification. An ether solution of the substrate (pheophytin or chlorophyll) was added to 0.08 m potassium phosphate buffer, pH 7.5, which was 0.2% with respect to the detergent Triton X-100. Although the residual ether could be removed under reduced pressure, the presence of 2% ether did not influence the reaction. A typical reaction mixture consisted of 20 to 50 mg of, or approximately 0.4 mM, substrate, 0.1 ml of enzyme solution or an equivalent amount of acetone powder, and 0.2% Triton-phosphate buffer to make 1.0 ml. The reaction mixture was incubated at 30°C in 12-ml graduated centrifuge tubes. The tubes were protected from light when light-sensitive substrates were used, and agitated whenever acetone powder was assayed.

At the conclusion of hydrolysis, 2 ml of acetone and 3 ml of petroleum ether (b.p. 60-90°) were quickly pipetted into the tubes which were then stoppered, shaken, and cleared by centrifugation. The unhydrolyzed substrate was removed into the petroleum ether layer, whereas the hydrolyzed portion remained in the aqueous acetone. The volumes of the two layers were recorded (usually 3.9 and 1.9 ml, respectively), the layers separated, and their optical densities at the λmax of the substrate (pheophytin a, 667 mp; pheophytin b, 655 mp) determined in a Beckman model DU spectrophotometer. For the calculation of pheophytin a concentrations, the specific absorption coefficients employed were: acetone 54.2, petroleum ether 62.6 liters per g cm. These values were determined experimentally. Parallel samples without added enzyme were carried through all steps to determine the extent of spontaneous hydrolysis and loss of substrate by decomposition in the course of the reaction. Enzyme activity may be expressed either in terms of disappearance of substrate or appearance of product. The agreement between these two modes of expression was quite close (Table I).

Chlorobium chlorophyll and bacteriochlorophyll are not soluble in petroleum ether and the above assays cannot, therefore, be applied to their hydrolysis by chlorophyllase. The reaction with these substrates was carried out as before, but at the conclusion all pigment was extracted into 6 ml of ether. The ether was then shaken with 0.02 N KOH which removed the liberated chlorophyllides. The degree of hydrolysis can then be determined by comparing the optical density of the ether solution with that of an identical sample carried through the same procedure without the addition of enzyme. This assay is not as satisfactory as the acetone-petroleum ether partition because a small amount of pigment cannot be extracted by ether from the detergent-containing reaction mixture. For this reason, the ether-KOH method was used only to confirm qualitatively

---

*This investigation was supported by grants from the National Science Foundation and the National Institutes of Health.
†Postdoctoral Fellow, National Cancer Institute, United States Public Health Service.
‡Seeds were purchased from Frank S. Platt Seed Company, Branford, Connecticut.
**Table I**

Assay of solubilized chlorophyllase in aqueous medium

Assay mixture consisted of 0.5 ml of substrate containing 39.5 µg of pheophytin a in 0.08 M phosphate buffer, pH 7.5, and 0.4% Triton X-100, 0.4 ml of the same phosphate buffer, and 0.1 ml of soluble enzyme preparation diluted 1:10 with distilled water. After incubation at 30°C, to each sample were added 2 ml of acetone and 3 ml of petroleum ether (b.p. 60-90°C), the tubes were shaken, cleared by centrifugation for 2 minutes, volumes of the two phases recorded, and their optical densities determined in a Beckman model DU spectrophotometer. Degree of hydrolysis was determined by decrease in O.D. at the petroleum ether phase (disappearance of substrate) and increase in O.D. of the aqueous acetone phase (appearance of product).

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Pheophytin hydrolyzed*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Based on disappearance</td>
<td>Based on appearance of product</td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------</td>
<td>---</td>
</tr>
<tr>
<td>10 min</td>
<td>1.45</td>
<td>1.25</td>
</tr>
<tr>
<td>2 hrs</td>
<td>11.5</td>
<td>11.3</td>
</tr>
<tr>
<td>3 hrs</td>
<td>17.1</td>
<td>17.1</td>
</tr>
<tr>
<td>5 hrs</td>
<td>25.7</td>
<td>25.6</td>
</tr>
<tr>
<td>10 hrs</td>
<td>32.0</td>
<td>32.0</td>
</tr>
<tr>
<td>24 hrs</td>
<td>33.0</td>
<td>32.7</td>
</tr>
</tbody>
</table>

* Values are averages from duplicate samples.

**Table II**

Procedure for solubilization of rye chlorophyllase

Fractions I and II were assayed as acetone powders. Fraction III was assayed both as acetone powder and in soluble form. Assay conditions were the same as in Table I.

<table>
<thead>
<tr>
<th>Step</th>
<th>Preparation</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity*</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Chloroplast suspension</td>
<td>100</td>
<td>875</td>
<td>2.2</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>SDC extract of butanol-treated chloroplasts</td>
<td>24</td>
<td>72</td>
<td>11</td>
<td>41</td>
</tr>
<tr>
<td>III</td>
<td>Buffer extract of ammonium sulfate precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a.</td>
<td>Acetone powder</td>
<td>3</td>
<td>0.5</td>
<td>390</td>
<td>19</td>
</tr>
<tr>
<td>b.</td>
<td>Soluble form</td>
<td>3</td>
<td>0.5</td>
<td>1080</td>
<td>28</td>
</tr>
</tbody>
</table>

* µg of pheophytin a hydrolyzed per hour per mg of protein.

**Figure 1.** Effect of substrate concentration on reaction rate. Assay conditions as described in Table I except for concentration of pheophytin a. Incubation was for 1 hour.

**Figure 2.** SUBSTRATE CONCENTRATION, M x 10⁻⁵

The presence of chlorophyllase activity against the two chlorophylls of bacterial origin. Protein content was determined by the method of Lowry et al. (7).

Preparation of Substrates —Chlorophyll a and pheophytin a as well as bacteriochlorophyll were prepared by sugar column chromatography according to Smith and Benitez (8). Chlorobium chlorophyll "650" was prepared by the method of Conti and Vishniac (9).

Reagents —Acetone, petroleum ether, and ether were redistilled in the laboratory.

RESULTS

Fractionation of Rye Extracts and Solubilization of Enzyme —The purification of chlorophyllase was worked out with etiolated rye seedlings as starting material. The very small amount of chlorophyll present in chloroplasts grown in the dark was completely removed in the course of enzyme purification.

I. Preparation of Particulate Cell Fraction —Rye seedlings were grown for 7 to 8 days at 24°C in the dark with daily tap water subirrigation. Chilled, etiolated leaves and stems, 400 g, were ground in a Waring Blender in 500 ml of 0.35 M NaCl. The pulp was strained through several layers of cheese cloth and centrifuged at 500 x g for 3 minutes to remove debris. The supernatant fluid was discarded. Although butanol alone did not solubilize chlorophyllase activity, this step was essential in the procedure. The rubbery pellet was next suspended in 30 ml of 1% SDC in the microblender, stored at 0°C for 30 minutes, and centrifuged at 20,000 x g for 20 minutes. The supernatant fluid from this centrifugation was a highly active preparation but more enzyme could be liberated by repeated SDC extraction. As a rule, three such extractions were made with 30, 20, and 20 ml of 1% SDC, and these extracts were pooled.

II. Solubilization of Chlorophyllase —The particulate residue from the 20,000 x g centrifugation was suspended in 50 ml of 10% i-butanol in a microblender and blended for 2 minutes. The slurry was centrifuged at 10,000 x g for 15 minutes, and the supernatant fluid was discarded. Although butanol alone did not solubilize chlorophyllase activity, this step was essential in the procedure. The rubbery pellet was next suspended in 30 ml of 1% SDC in the microblender, stored at 0°C for 30 minutes, and centrifuged at 20,000 x g for 20 minutes. The supernatant fluid from this centrifugation was a highly active preparation but more enzyme could be liberated by repeated SDC extraction. As a rule, three such extractions were made with 30, 20, and 20 ml of 1% SDC, and these extracts were pooled.

III. Ammonium Sulfate Precipitation —The milky SDC extract was made 30% saturated with respect to ammonium sulfate. After 30 minutes at 0°C, the precipitate was collected by centrifugation and washed with an ammonium sulfate solution of the same strength. The residue was extracted three times with

2 We wish to thank Dr. Sam Conti of Brookhaven National Laboratory for generously supplying lyophilized Chromatium and Chlorobium cells.

The abbreviations used are: SDC, sodium deoxycholate; DFP, diisopropyl fluorophosphate.
Aqueous Chlorophyllase Systems

Vol. 236, No. 9

HOURS OF INCUBATION

FIG. 2. Time course of hydrolysis and equilibrium point. Assay conditions as described in Table I. Amount of pheophytin a in each sample was 39.5 µg. At equilibrium, 85.9% of substrate was hydrolyzed.

ENZYME CONC. (µg PROTEIN)

Fig. 3. Effect of enzyme concentration. Assay conditions as described in Table I. Amount of pheophytin a was 39.5 µg per sample. Incubation was for 1 hour.

2 ml portions of phosphate buffer (0.1 M, pH 7.5). The final preparation was a clear, yellow solution with a specific chlorophyllase activity 500-fold greater than that of the original pulp. It could be frozen and thawed repeatedly as well as stored in the frozen state for several months without significant loss of activity. The formation of a SDC-protein complex was suggested by the formation of a clear gel when the preparation was cooled to 0°. Dialysis against water resulted in a precipitate and loss of activity, whereas dialysis against 0.04% SDC did not affect the soluble enzyme (summarized in Table II).

Effect of pH—Within the pH range of 6 to 8, the activity of the enzyme remained constant. Below pH 6, the activity fell off rapidly.

Influence of Substrate Concentration—The effect of pheophytin a concentration on the velocity of hydrolysis is shown in Fig. 1. A Michaelis-Menten constant of $1.5 \times 10^{-5}$ was obtained and confirmed by a Lineweaver-Burk plot.

Time Course of Hydrolysis—In Fig. 2, the progress of hydrolysis of pheophytin a with time is plotted. It may be noted that the equilibrium point of 85% hydrolysis is approached after 10 hours of incubation with only slight further hydrolysis taking place in the ensuing 38 hours. The position of the equilibrium point of 85% hydrolysis was confirmed by other long term experiments.

Effect of Enzyme Concentration on Reaction Rate.—Data presented in Fig. 3 indicate that within the range tested the reaction obeys first order kinetics with respect to enzyme.

Effect of Inhibitors—In Table III, the action of several inhibitors on chlorophyllase is presented. Particularly noteworthy is the absence of any DFP inhibition. The other data point to the presence of a sulfhydryl group necessary for chlorophyllase action.

Specificity—Qualitative tests were performed to check the effectiveness of rye chlorophyllase against several substrates other than pheophytin a on which the bulk of the work here reported was done. Pheophytin b, chlorophyll a, bacteriochlorophyll, and chlorobium chlorophyll were all hydrolyzed.

Reversibility—Although Wilstätter, in his original description of chlorophyllase, reported that the hydrolysis reaction was reversible, no subsequent investigation on this aspect of the enzyme is available in the literature. Our attempt to reverse the reaction in the standard assay mixture was unsuccessful.

DISCUSSION

The present investigation confirms the conclusion of Ardao and Vennesland (3) that chlorophyllase is intimately tied to a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None ..................................................................</td>
<td>100</td>
</tr>
<tr>
<td>$p$-Chloromercuribenzoate, $5 \times 10^{-4}$ M ........</td>
<td>0</td>
</tr>
<tr>
<td>$p$-Chloromercuribenzoate, $5 \times 10^{-4}$ M + GSH, $5 \times 10^{-4}$ M</td>
<td>88</td>
</tr>
<tr>
<td>$p$-Chloromercuribenzoate, $5 \times 10^{-4}$ M, followed by GSH, $5 \times 10^{-4}$ M</td>
<td>57</td>
</tr>
<tr>
<td>Sodium ferricyanide, $2 \times 10^{-3}$ M ..................</td>
<td>83</td>
</tr>
<tr>
<td>Iodoacetamide, $3.3 \times 10^{-3}$ M ..................</td>
<td>100</td>
</tr>
<tr>
<td>DFP $4 \times 10^{-3}$ M ..................................</td>
<td>100</td>
</tr>
</tbody>
</table>
water-insoluble lipoprotein fraction of chloroplasts (chloroplas- tin). The formation of a complex between this fraction and SDC permits the characterization of chlorophyllase in an aqueous medium. The same authors (3) have attributed the inactivity of digitonin-solubilized chloroplas tin against aqueous suspensions of chlorophyll to a lack of contact between enzyme and substrate. In our assay procedure, substrates are made accessible by the detergent action of Triton X-100.

The absence of a sharp pH optimum for chlorophyllase action may be attributed to protection of enzyme surfaces by the SDC forming a complex. It is also possible that the active site which is capable of accommodating phytol is generally lipophilic and, thus, less subject to variations of pH in the aqueous medium. Several seed lipases show similarly weak pH dependence (10).

The method of preparation and assay reported here permits a study of the role that this enzyme plays in the chloroplast. Although chlorophyllase is likely to participate in the biosynthesis of chlorophyll, we have failed to demonstrate esterification with phytol under our conditions. Perhaps the reaction could be facilitated by providing a methyl or ethyl chlorophyllide instead of the free acid. It would also be important to investigate whether this enzyme can carry out the phytylation of protochlorophyllide. It has been suggested (11) that it is at the level of chlorophyllide in the biosynthesis of chlorophyll that esterification takes place, although there is evidence for the existence of phytilated protochlorophyllide as well (12).

Butler (13) has suggested that chlorophyllase may play a major role in the synthesis of chloroplast lamellas, and it will, therefore, be important to determine the localization of the enzyme in the developing chloroplast. The presence of chlorophyllase in a tightly bound insoluble form in etiolated plastids would suggest its association with rudimentary lamellas or crystalline centers (14) as well as with the “grana” which develop in the light. Such studies might be carried out with mutants defective in chloroplast structure. Finally, it would be of interest to determine the sequence of events in plastid senescence. Does the release and hydrolysis of chlorophyll proceed the breakdown of the lamellas, or is it the breakdown that makes chlorophyll accessible to chlorophyllase?

**SUMMARY**

A procedure is described for the 500 fold purification of chlorophyllase from etiolated rye seedlings. Evidence is presented that the final preparation is a lipoprotein-sodium deoxycholate complex. An assay procedure with an aqueous medium is presented. p-Chloromercuribenzoate and ferricyanide inhibit the enzyme, whereas iodoacetamide and diisopropyl fluorophosphate do not, at the concentrations tested. The possible role of the enzyme in the biosynthesis and breakdown of chlorophyll and in the formation of lamellas of the chloroplast is discussed.

**REFERENCES**

2. KROESSING, G., Biochem. Z., 305, 359 (1940).