Chlorophyllase

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I. INTRODUCTION

Chlorophyllase (EC 3.1.1.14) was first described in 1913 (1). When attempts have been made to detect it, chlorophyllase has been found in all green plants and algae. Chlorophyllase can hydrolyze alcohols esterified to the propionic acid side chain on C_7 of the porphyrin ring of chlorophylls, pheophytins, and various derivatives of these compounds. In addition, it can catalyze transesterification reactions (2) that result in the addition of a variety of side chains to the porphyrin ring. See Table 1 and Figure 1 for the nomenclature of chlorophyllase substrates. A previous review of this enzyme has been published by Drazkiewicz (3).

II. MEASUREMENT OF CHLOROPHYLLASE ACTIVITY

Chlorophylls or pheophytins are the usual substrates for chlorophyllase activity assays. These compounds are insoluble in water. Therefore, either reactions must be done with the substrate solubilized into an aqueous buffer system using a surfactant, or there must be sufficient organic solvent, usually acetone, added to the reaction mixture to dissolve the substrate. McFeeters et al. (4) provided an example of the aqueous buffer system adapted from the earlier procedure of Klein and Vishniac (5). Examples of the use of acetone to solubilize substrates are Weast and Mackinney

Table 1 Structures for Substrates and Inhibitors of Chlorophyllase[a]

Compound	Mg^{2+} (±)	7, 8 Position reduced (±)	R	R_1	R_2	R_3	R_4
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
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
9-Hydroxyl methyl pheophorbide *a*	−	+	CH_3	CH_2CH_3	OH	CO_2CH_3	CH_3
Pyropheophytin *a*	−	+	CH_3	CH_2CH_3	=O	H	phytyl
[I]Protochlorophyll *a*	+	−	CH_3	CH_2CH_3	=O	CO_2CH_3	phytyl
[I]4-Vinyl protochlorophyll *a*	+	−	CH_3	$CH{=}CH_3$	=O	CO_2CH_3	phytyl

[a] See Figure 1 for the ring structure common to these compounds
[I] Inhibitor

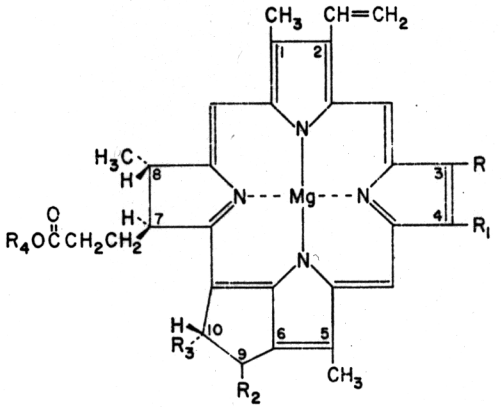

Figure 1 Ring structure for compounds that are substrates or inhibitors of chlorophyllase

(6) and Shioi et al. (7). The reaction is stopped, and the product is separated from the remaining substrate by partitioning between an organic phase and an aqueous acetone phase after KOH solution is added to raise the pH. For measurement of pheophytin or chlorophyll hydrolysis, McFeeters et al. (4) added 60% hexane:40% acetone and KOH solution to raise the reaction mixture pH to 8.5. The final proportion of solvents was 50% hexane, 33.3% acetone, and 16.7% water. After separation of the two phases, substrate depletion or, preferably, product formation is measured spectrophotometrically. To measure hydrolysis of methyl and ethyl chlorophyllides or pheophorbides, McFeeters (8) used a mixture of hexane, acetone, and 2-butanone to separate reaction products from the substrates.

III. PURIFICATION

Chlorophyllase was first purified to homogeneity by Moll and Stegwee (9). Subsequently, Shioi et al. (7) developed a somewhat simplified method. A homogeneous preparation of the enzyme was prepared from *Chlorella protothecoides* with over a 50% yield using a butanol extraction, ammonium sulfate precipitation, and chromatography on size exclusion columns (Table 2). The same procedure, except that the butanol solubilization step was unnecessary, resulted in purification of the enzyme from tea leaf sprouts but with only a 9.6% yield (10). Trebitsch et al. (11) purified chlorophyllase from the flavedo of mature green oranges, determined the N-terminal sequence of the purified enzyme, and used it for generation of antibodies to chlorophyllase. Tauchiya et al. (12) purified two chlorophyllase isozymes from mature *Chenopodium album* leaves using hydrophobic chromatography, ConA Sepharose, heparin, and ion exchange HPLC on two different columns. The presence of isozymes was confirmed by N-terminal sequencing of the separated chlorophyllases and by showing that the 10th amino acid from the N-terminus was different (Fig. 2). However, the N-terminal sequences of the *Chenopodium* isozymes had no homology with the sequence from orange chlorophyllase (11) or with any other published protein sequence (Fig. 2).

IV. PROPERTIES

The molecular weight for chlorophyllase has been reported to range from 27 to 65 kDa. Chlorophyllase from citrus fruit peel was found to be 27 kDa in one study (13) and 35 kDa in another (11). *Phaeodactylum* was reported to have two enzymes that were 43 and 46 kDa in size (14). Isozymes from *Chenopodium album* were 41.3 and 40.2 kDa (12). The largest size chlorophyllase reported to date was 65 kDa in *Chlorella regularis* (15). Most frequently, however, molecular size has been found to be near 38 kDa. This includes the chlorophyllase from *Chlorella protothecoides* (7), tea leaf sprouts (10), sugar beet leaves (16), and rye seedlings (17).

Most often, chlorophyllases have been found to have pH optima near pH 7. However, McFeeters et al. (4) and Ogura (18) found chlorophyllases from *Ailanthus altissima* and tea, respectively, had acidic optima. McFeeters et al. (4) showed a pH optimum near 7 could be obtained erroneously if the pH of the aqueous phase in the usual organic solvent/aqueous partitioning mixtures was not raised by the addition of KOH. Therefore, earlier reports of pH optima for chlorophyllases may be in error. This work indicated the absence of ionizable groups in the enzyme that affected substrate binding, but there did appear to be a pK_a 3.4 group in the active site of the enzyme involved in substrate hydrolysis. Use of acetone in the reaction mixture when determining the optimum pH of chlorophyllase could raise the apparent pK_a of this group and give a higher pH optimum in the presence of acetone than in the absence of this solvent.

The effect of different substituents of the chlorophyll *a* molecule on the ability of chlorophyllase to bind and hydrolyze potential substrates has been investigated. The picture that emerges is that of a rather high specificity esterase. First, chlorophyllase does not require the presence of a metal ion in the center

Table 2 Purification of Chlorophyllase from *Chlorella protothecoides*[a]

Purification step	Protein (mg)	Total activity (units)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)
Butanol extract	1105	1436	1.3	1	100
Ammonium sulfate (0–30% saturation)	12.4	1561	126	97	109
First Sephadex G-200	5.50	1359	247	190	95
Sepharose CL-6B	1.31	902	689	530	63
Second Sephadex G-200	0.79	758	960	738	53

[a] Chlorophyllase was extracted from 50 g (wet weight) of cells.
Source: Ref. 7.

of the tetrapyrrole ring. This is demonstrated by the fact that chlorophyllase has been found to hydrolyze chlorophylls and pheophytins with similar K_m and V_{max} value (4). Kinetic data suggested that the K_m is a binding constant for those substrates. Data have not been obtained to determine the effect of different metal ions in the porphyrin ring on chlorophyllase hydrolysis. Transesterification experiments have shown that chlorophyllase can use a variety of primary and secondary alcohols in place of phytol alcohols as substrates (2). This implies that the enzyme can also hydrolyze chlorophyllide esters of the same alcohols. Protochlorophyll and 1-vinyl protochlorophyll are not hydrolyzed by chlorophyllases, but they bind with affinities similar to pheophytin *a* (4). This showed that a double bond between C_7 and C_8 does not affect binding to chlorophyllase but that it prevents hydrolysis of phytol. Similarly, removal of the carbomethoxy group from C_{10} of pheophytin greatly reduces hydrolysis rates with little effect upon binding (4). Fiedor et al. (19) found that chlorophylls a′ and b′ were not hydrolyzed by chlorophyllase from two different plants and that the presence of these isomers did not affect the hydrolysis rates of the corresponding chlorophylls. These results suggested that a carbomethoxy group on the opposite side of ring V, as occurs in chlorophyll, prevents both binding and hydrolysis of chlorophylls a′ and b′. Comparison of the kinetics of hydrolysis of methyl pheophorbide *a* with 9-hydroxy methyl pheophorbide *a* showed that introduction of a hydroxyl group at C_9 in ring V reduced the binding affinity of substrate dramatically while, at the same time, having little effect on substrate hydrolysis rate (8).

There is little information available on the stability of chlorophyllase from different plants. In general, assays for activity have been performed at 30°C at a pH near 7. Recently, a 30°C optimal temperature was reported for chlorophyll hydrolysis by crude chlorophyllase extracts from artichoke (20). Chlorophyllase activity was nearly inactivated during a 1-h incubation

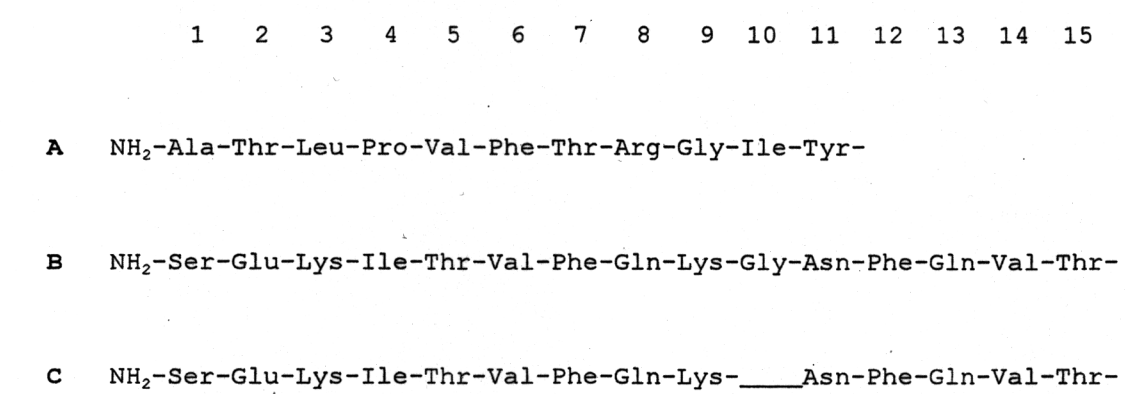

Figure 2 N-terminal sequences of chlorophyllases from orange (*Citrus sinensis* L.) (A) and *Chenopodium album* (B and C). (From Refs. 11, 12.)

at 45°C. Partially purified chlorophyllase from *Ailanthus altissima* retained > 90% of its activity during a 1-h incubation between pH 3.7 and 9.2 (4).

V. FUNCTION IN CHLOROPHYLL METABOLISM

There has been uncertainty for many years whether chlorophyllase is active in chlorophyll biosynthesis or degradation in plants. However, Rudiger et al. (21) have demonstrated the existence of a chlorophyll synthase which catalyzes esterification of chlorophyll using geranyl geranyl pyrophosphate as the substrate. The geranyl geranyl ester is then reduced to phytol, the usual side chain in the chlorophylls of higher plants. There is the possibility that "chlorophyll synthase" and "chlorophyllase" are two names given to the same enzyme investigated in different ways, as was assumed by Dogbo et al. (22).

However, the information available on the substrate specificity of chlorophyllase described above compared to the substrate specificity of chlorophyll synthase activity strongly suggests that these are different enzymes. For example, pheophorbide *a* is not a substrate for chlorophyll synthase (23), but the pheophytins are readily hydrolyzed to pheophorbides by chlorophyllase (4). In addition, pyrochlorophyllide *a* is a good substrate for chlorophyll synthase (24), but the V_{max} for pyropheophytin hydrolysis by chlorophyllase is only 2.5% as large as the V_{max} for pheophytin hydrolysis (8). Langmeier et al. (25) concluded there is little double that chlorophyllase is indispensable for chlorophyll degradation. This was based upon evidence that pheophorbide *a* is a required intermediate in the degradation of chlorophyll to fluorescent linear tetrapyrrole compounds. However, it may not be the first enzyme in the degradative pathway because Mg^{2+} removal may occur prior to phytol hydrolysis (25).

VI. LOCATION AND PHYSIOLOGICAL CHANGES

Brandis et al. (26) found chlorophyllase to be associated with the chloroplast envelope and not with chlorophyll–protein complexes, as has been reported earlier (27). This has been confirmed by Matile et al. (28). They also found that pheophorbide *a* oxygenase, the enzyme responsible for degrading the cyclic tetrapyrrole chlorophyll degradation product to a linear tetrapyrrole, is located in the chloroplast envelope. They suggest that the initiation of chlorophyll degradation must involve a mechanism to transport chlorophyll from the thylakoid pigment–protein complexes to the chloroplast envelope where chlorophyllase is located.

Trebitsh et al. (11), using chlorophyllase antibodies to detect chlorophyllase molecules, found that chlorophyllase was synthesized de novo when green oranges were exposed to ethylene to speed ripening. The senescence-delaying plant regulators gibberellin A_3 and N^6-benzyladenine inhibited the synthesis of chlorophyllase, which could be induced by ethylene.

VII. SIGNIFICANCE IN FOOD PROCESSING

Chlorophyllase appears to be of limited significance in food processing and storage. Dephytylated chlorophyll derivatives, particularly pyropheophorbide *a*, have been implicated in photosensitization reactions in humans. It has been observed that higher levels of these derivatives in food supplements prepared from leaf protein concentrate (29) or dried *Chlorella* cells (30) is correlated with chlorophyllase activity in the plant or alga.

Pheophorbides derived from chlorophylls have been observed in pickles (31), fermented olives (32), and coleslaw (33). Modeling of these data suggested that, in coleslaw, chlorophyllase converted pheophytins to pheophorbides after the chlorophylls were initially converted to pheophytins by the low pH (34). In the fermented vegetables, chlorophylls were first converted to chlorophyllides by chlorophyllase, and, as acid formed during fermentation, the chlorophylides converted to pheophorbides (34). While pheophytins and the corresponding pheophorbides have the same visible spectra in organic solvents, Heaton et al. (33) observed that the color of coleslaw changed as chlorophyllase hydrolyzed pheophytins to the corresponding pheophorbides.

Efforts have been made to promote retention of green color in processed vegetables by treating them to increase formation of chlorophyllides from chlorophylls (35). This was done on the assumption that magnesium ions would be removed more slowly from chlorophyllides than chlorophylls. However, it has been shown that chlorophyllides lose the magnesium ion more rapidly than do chlorophylls (36). Ihl et al. (20) found that inactivation of chlorophyllase in green artichokes correlated with optimum color retention for microwave and boiling-water blanching procedures.

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