Rapid communication

Localization of chlorophyllase in the chloroplast envelope

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Abstract. Chlorophyllase catalyzes the first step in the catabolic pathway of chlorophyll. It is a constitutive enzyme located in chloroplast membranes. In isolated plastids the hydrolysis of the endogenous chlorophyll does not take place unless the membranes are solubilized in the presence of detergent. The structural latency of chlorophyllase activity appears to be due to the differential locations of substrate and enzyme within the plastids. Envelope membranes prepared from both chloroplasts and gerontoplasts contain chlorophyllase activity. The isolation of envelopes is associated with a marked increase in chlorophyllase activity per unit of protein. Yields of chlorophyllase and of specific envelope markers in the final preparations are similar, suggesting that the enzyme may be located in the envelope. It is hypothesized that the breakdown of chlorophyll during leaf senescence requires a mechanism that mediates the transfer of chlorophyll from the thylakoidal pigment-protein complexes to the sites of catabolic reactions in the envelope.

Key words: *Brassica* – Chlorophyllase – Chloroplast envelope – *Hordeum*

The existence in green leaves of an esterase that hydrolyses chlorophyll (Chl) into chlorophyllide (Chlide) and phytol has been known for more than 80 years: Stoll (1912) not only discovered chlorophyllase (E.C. 3.1.1.14) to be present in green leaves of all species examined, he also observed that the cleavage of the endogenous Chl did not take place unless the leaves were treated with ethanol or moist ether. The effect of such treatments suggests that in the living cells the enzyme is structurally latent.

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Abbreviations: ACT=acyl CoA thioesterase; Chl=chlorophyll; Chlide=chlorophyllide; PC=phosphatidylcholine

Krossing (1940) was first in demonstrating the presence of chlorophyllase in a chloroplast fraction and since then the association of chlorophyllase with particles and pigment-protein complexes prepared in various ways from chloroplast membranes has been confirmed repeatedly (Ardao and Vennesland 1960; Terpstra 1974; 1976; Tarasenko et al. 1986). It has also been observed that even in isolated pigment-protein complexes chlorophyllase remains latent; indeed, the hydrolysis of the endogenous Chl requires the presence of either appropriate detergents (Amir-Shapira et al. 1986) or acetone (Garcia and Galindo 1991).

Attempts to establish the exact association of chlorophyllase with specific pigment-protein complexes have yielded ambiguous results. On the one hand chlorophyllase has been detected in components of the light-harvesting complex of PSII (LHC II) that were separated electrophoretically (Tarasenko et al. 1986; Brandis et al. 1996) whilst, on the other, attempts to separate solubilized complexes by density gradient centrifugation have failed to confirm an intimate association of chlorophyllase and LHC II (Schellenberg and Matile 1995; Brandis et al. 1996). Moreover, purified LHC II has been found to be devoid of chlorophyllase activity (Schellenberg and Matile 1995).

Hirschfeld and Goldschmidt (1983) have shown that chlorophyllase is present in Chl-free chromoplast membranes prepared from *Citrus* fruits. As thylakoids are cleared away upon the transformation of chloroplasts into chromoplasts, this finding suggests that chlorophyllase may be located in the plastid envelope rather than in the thylakoids. Such a differential location in chloroplasts of chlorophyllase in the envelope and Chl in the thylakoids would provide a plausible explanation of latency.

Since chlorophyllase must be considered to represent the first enzyme in the catabolic pathway of Chl, its exact localization is decisive for understanding the regulation of breakdown. We therefore decided to explore the putative location through direct analysis of isolated chloroplast envelopes.

Plant material. Seedlings of barley (*Hordeum vulgare* L. cv. Express) and oilseed rape (*Brassica napus* L. cv. Arabella) were purchased from Volg (Winterthur, Switzerland) and cultivated as detailed previously (Hörtensteiner et al. 1995). Senescence was induced by placing excised shoots in darkness at room temperature. Shoots of barley were harvested on day 10 after sowing, those of rape on day 12.

Preparation of envelopes. In the case of barley the method developed by Douce and Joyard (1982) was employed. A detailed description of the preparation of intact plastids has been reported elsewhere (Matile et al. 1992).

For the isolation of envelope membranes, Schünemann and Borchert (1994) have recently used conventional homogenates instead of intact chloroplasts. They observed that envelope membranes are present among the particulate material of the supernatant obtained upon the sedimentation of broken chloroplasts. However, the separation by density gradient centrifugation of envelope membranes from mitochondrial and microsomal contaminations was unsatisfactory. We observed that envelope membranes still associated with broken chloroplasts prepared from conventional homogenates can partially be detached and hence contamination be avoided' as described in the following.

Rape cotyledons (20 g FW) were blended (2×5 s; omnimixer, max. speed; Sorvall, Newtown, Conn. USA) in the presence of 100 mL medium [0.6 M sorbitol, 20 mM Tris-N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine) buffer (pH 8), 2 mM dithiothreitol (DTT), 10 mM Na ascorbate, 5 mM cysteamine, 5 mM polyethyleneglycol 4000, 0.1% bovine serum albumin]. The filtered homogenate was centrifuged at 7700·g for 5 min to yield a green pellet of broken chloroplasts which was resuspended and washed once in 40 mL buffered sorbitol medium containing 5 mM DTT and 5 mM ascorbate. To detach envelope and thylakoid membranes the chloroplasts were suspended in 4.4 mL buffer (10 mL Tris-Tricine, pH 8; 2 mM DTT) and gently agitated (0° C,darkness). A crude preparation of envelope membranes was obtained by layering 1-mL aliquots onto 2-mL sorbitol cushions (0.7 M sorbitol, 10 mM buffer, 2 mM DTT) and sedimenting the thylakoid-rich material (4 min, 2000·g). Upon the removal of the supernatant, the green sediment was resuspended anew in buffer and the suspension run through the same procedure. Supernatants collected and combined from a total of three repetitions were centrifuged (1 h, 39000·g) to yield a slightly greenish sediment. Upon resuspension in 3.5 mL buffer the preparation was loaded onto a stepwise sucrose gradient (5 mL of 1.0 M, 10 mL of 0.8 M, 10 mL of 0.3 M, 5 mL of 0.2 M buffered sucrose) and centrifuged (60 min, 95000·g). Envelope membranes trapped at the 0.8/0.3 M interface were collected, diluted three times with buffer, pelleted (50 min, 16000·g) and resuspended in 800 μL buffer. The other fractions of the gradient (interfaces and green pellet) were converted into preparations of defined volumes and used for calculations of recoveries.

Determination of enzyme activities. Chlorophyllase from barley was optimally active in the present of 1% (w/v) Triton X-100, whereas in the case of rape the addition of acetone (22%, v/v) was required. The incubation mixture for barley consisted of 160 μL enzyme preparation; 20 μL of 0.2 M phosphate buffer (pH 8) containing 10% Triton X-100; 5 mM MgCl₂; and 20 μL substrate containing 40 μg Chl. The incubation mixture for rape consisted of 20 μL enzyme preparation; 11 μL of 10 mM Tris-Tricine buffer (pH 8); 2 mM DTT; 9 μL acetone; and 2 μL substrate containing 7 μg Chl dissolved in 20% Triton-X-100. In both cases the incubations (barley: 60 min; 40° C; rape: 30 min, 20° C) were stopped by the addition of acetone (ad 80%, v/v) followed by the extraction of Chl with hexane and the analysis of Chlides in the aqueous phase (barley: spectrophotometry at 667 nm; rape: HPLC).

Phaeophorbide *a* oxygenase activity was determined as outlined in Hörtensteiner et al. (1995).

Acyl CoA thioesterase (ACT) was measured according to Schünemann and Borchert (1994) with [1-¹⁴C]palmitoyl CoA as substrate.

Determination of phosphatidylcholine (PC) protein and Chl. The analysis of PC has been described previously (Matile and Schellenberg 1996). Chlorophyll was determined using the coefficients provided by Lichtenthaler (1987) and protein-dye binding (Bradford 1976) was employed for quantitation of protein.

The structural latency of chlorophyllase in chloroplasts prepared from barley mesophyll is illustrated in Fig. 1. In the intact membranes and even after subjection of the membranes to freezing and thawing, the Chl was stable. The contact between the endogenous Chl and chlorophyllase was only achieved when the membranes were solubilized in the presence of Triton X-100 at sufficient concentrations. Structural latency of chlorophyllase as shown in Fig. 1 could be equally demonstrated in chloroplast membranes from mature as well as senescent leaves.

To test whether latency of chlorophyllase is due to differential location of enzyme and substrate within chloroplasts, envelope preparations were analyzed for chlorophyllase activity. The results obtained with membranes from barley chloroplasts are compiled in Table 1. They clearly demonstrate that chlorophyllase activity was consistently present in the Chl-free envelope preparations. Upon the isolation of envelopes the specific activities (per protein) increases 10- to 20-fold compared with those in the lysed chloroplasts and gerontoplasts (senescent chloroplasts).

Since the purification of envelope membranes yields rather small proportions of the total membranes present
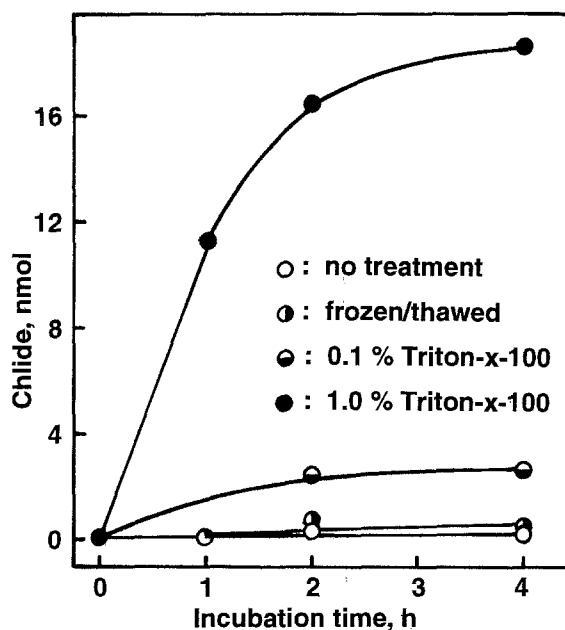

Fig. 1. Demonstration of latency of chlorophyllase activity in chloroplast membranes from presenescent barley primary leaves. The membrane equivalent of 50 mg leaf FW' containing 36 μg of endogenous Chl' was suspended in 200 μL isolation medium (0.6 M sorbitol, 10 mM Tris-Tricine buffer, pH 8) containing detergent as indicated' and incubated at 40° C in darkness. The membranes were either not pretreated or subjected to one cycle of freezing and thawing prior to incubation. Reactions were terminated by the addition of 800 μL acetone. Chlorophyllides produced were determined spectrophotometrically at 663 nm after extraction of residual Chl with petroleum ether

in the preparations of lysed plastids, it was important to quantify yields by employing markers of the envelope membranes. Thus, PC was taken as a marker compound of the outer (Douce and Joyard 1990), ACT as a marker enzyme of the inner envelope membrane (Schünemann and Borchert 1994). Yields of chlorophyllase activity in the envelope preparations were always similar to those of ACT activity, whereas in nine out of a total of ten independet isolations, yields of PC were markedly lower. According to Douce and Joyard (1982) the lysis of chloroplasts results in the disruption of the envelope and the formation of vesicles composed of inner and outer envelope membranes. The different yields of the two markers suggest that these vesicles may contain a comparatively high proportion of the inner membrane which is marked by the activity of ACT.

With regard to the regulation of Chl breakdown it was important to find out whether chlorophyllase retains its location when chloroplasts are induced to break down

Table 1. Localization of chlorophyllase (Chlase) activity in envelope membranes isolated from barley chloroplasts (Chpls) and gerontoplasts (Gpls). Chloroplasts were obtained from primary leaves harvested on day 10 after sowing (No. 1). For the preparation of gerontoplasts, excised leaves were induced to senesce in darkness for 4 d (No. 2) or 6 d (No. 3)

No.	Preparations	Chlase activity		Envelope markers		Chl[a]
		Relative[a]	Specific[b]	PC[a]	ACT activity[a]	
1	Lysed Chlpls	100	73	100	100	100
	Envelopes	6	703	8	5	<0.1
	Recovery[c]	95	–	91	n.d.	98
2	Lysed Gpls	100	35	100	100	100
	Envelopes	16	480	3	12	0
	Recovery[c]	123	–	72	n.d.	90
3	Lysed Gpls	100	80	100	100	100
	Envelopes	10	1552	2	8	<0.1
	Recovery[c]	113	–	61	n.d.	114

[a] Activities/amount in the lysed plastids taken as 100
[b] nmol Chl·(mg protein)$^{-1}$·h^{-1}
[c] Sum of activities/amounts measured in all fractions obtained upon the separation of envelope membranes calculated as a percentage of those present in the lysed plastids. Chlorophyllase activities not contained in the preparations of envelopes were recovered in the Chl-containing fractions
n.d., not determined

Chl. As shown in Table 1 the location of chlorophyllase is unchanged during differentiation of chloroplasts into gerontoplasts. This finding is consistent with the observation that in membranes of gerontoplasts, chlorophyllase is as latent with regard to the endogenous Chl as it is in chloroplasts.

It has previously been shown that another enzyme engaged in Chl breakdown, phaeophorbide a oxygenase which is responsible for the cleavage of the porphyrin macrocycle (Hörtensteiner et al. 1995), is also located in the envelope isolated from barley gerontoplasts (Matile and Schellenberg 1996). This finding was confirmed upon the analysis of envelopes prepared from rape gerontoplasts (Table 2). Yields of chlorophyllase and oxygenase activities were similar to yields of the two envelope markers employed, suggesting that both enzymes may be located in the envelope of rape gerontoplasts. In a total number of five independent isolations the yields were consistently higher than those achieved with the method of Douce and Joyard (1982) but the purity of preparations was somewhat less satisfactory as indicated by the comparatively high contamination with Chl, i.e. thylakoids (Table 2).

In contrast to chlorophyllase, phaeophorbide a oxygenase is not constitutive but regulated in a sensescence-specific fashion. It is quite obvious that the oxygenase, which in the catabolic pathway acts downstream of the chlorophyllase, cannot be responsible for the overall control of Chl breakdown in senescent leaves. Therefore, a decisive element that is still missing concerns the mediation during leaf senescence of contact between the thylakoidal Chl and the chlorophyllase which seems to reside in the envelope. Results obtained with leaves of a mutant genotype of *Festuca pratensis* that is deficient with regard to phaeophorbide a oxygenase (Vicentini et al. 1995) may indicate the nature of the missing element. When leaves of the mutant were induced to senesce they stayed green but the first two intermediates of Chl breakdown, Chlides and phaeophorbide a, were accumulated progressively (Thomas et al. 1989). Although both enzymes involved, chlorophyllase and Mg-dechelatase, are constitutive (Vicentini et al. 1995), they remained inactive when cytoplasmic protein synthesis was inhibited by cycloheximide (Thomas et al. 1989). It appears, therefore, that the abolishment of latency of chlorophyllase in vivo requires the action of newly synthesized senescence-specific protein(s). We hypothesize that these pro-

Table 2. Localization of chlorophyllase (Chlase) and phaeophorbide a oxygenase activities in envelope membranes prepared from rape cotyledons. Results from two independent fractionations. Broken gerontoplasts (Gpls) were obtained from cotyledons induced to senesce in darkness for 4 d

No.	Preparation	Chlase activity		Phaeide a oxygenase Relative[a]	Envelope markers		Chl[a]
		Relative[a]	Specific[b]		PC[a]	ACT activity[a]	
1	Broken Gpls	100	7.1	100	100	100	100
	Envelopes	30	79.3	21	24	26	0.5
2	Broken Gpls	100	6.0	100	100	100	100
	Envelopes	17	72.0	13	20	18	0.7

[a] Enzyme activities and amounts, respectively, in gerontoplasts taken as 100
[b] nmol Chl·(mg protein)$^{-1}$·h^{-1}

teins have a function in picking up Chl molecules from thylakoidal pigment-protein complexes and transporting them to the sites of breakdown in the inner envelope membrane. The occurrence in senescent chloroplasts of such carrier protein for Chl destined to be degraded is presently being investigated.

References

Amir-Shapira D, Goldschmidt EE, Altman A (1986) Autolysis of chlorophyll in aqueous and detergent suspensions of chloroplast fragments. Plant Sci 43:201–206

Ardao C, Vennesland B (1960) Chlorophyllase activity of spinach chloroplastin. Plant Physiol 35:368–371

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brandis A, Vainstein A, Goldschmidt EE (1996) Distribution of chlorophyllase among components of chloroplast membranes in orange (Citrus sinensis) leaves. Plant Physiol Biochem 34:49–54

Douce R, Joyard J (1982) Purification of the chloroplast envelope. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier Biomedical Press, Amsterdam, New York, Oxford, pp 239–256

Douce R, Joyard J (1990) Biochemistry and function of the plastid envelope. Annu Rev Cell Biol 6:173–216

Gracia AL, Galindo L (1991) Chlorophyllase in Citrus leaves. Localization and partial purification of the enzyme. Photosynthetica 25:105–111

Hirschfeld KR, Goldschmidt EE (1983) Chlorophyllase activity in chlorophyll-free Citrus chromoplasts. Plant Cell Rep 2:117–118

Hörtensteiner S, Vincentini F, Matile Ph (1995) Chlorophyll breakdown in senescent cotyledons of rape, Brassica napus

L.: enzymatic cleavage of phaeophorbide a in vitro. New Phytol 129:237–246

Krossing G (1940) Versuche zur Lokalisation einiger Fermente in den verschiedenen Zellbestandteilen der Spinatblätter. Biochem Z 305:359–373

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Matile Ph, Schellenberg M (1996) The cleavage of phaeophorbide a is located in the envelope of barley gerontoplasts. Plant Physiol Biochem 34:55–59

Matile Ph, Schellenberg M, Peisker P (1992) Production and release of a chlorophyll catabolite in isolated senescent chloroplasts. Planta 187:230–235

Schellenberg M, Matile Ph (1995) Association of components of the chlorophyll catabolic system with pigment-protein complexes from solubilized chloroplast membranes. J Plant Physiol 146:604–608

Schünemann D, Borchert S (1994) Specific transport of inorganic phosphate and C3- and C6-sugar phosphates across the envelope membranes of tomato (Lycopersicon esculentum) leaf-chloroplasts, tomato fruit-chloroplasts and fruit-chromoplasts. Bot Acta 107:461–467

Stoll A (1912) Über Chlorophyllase und die Chlorophyllide. PhD thesis, ETH Zürich, Nr. 49

Tarasenko LG, Khodasevich EV, Orlovskaya KI (1986) Location of chlorophyllase in chloroplast membranes. Photobiochem Photobiophys 12:119–121

Terpstra W (1974) Properties of chloroplasts and chloroplast fragments as deduced from internal chlorophyll-chlorophyllide conversion. Z Pflanzenphysiol 71:129–143

Terpstra W (1976) Chlorophyllase and lamellar structure in Phaeodactylum triconutum III. Situation of chlorophyllase in pigment membranes. Z Pflanzenphysiol 80:177–188

Thomas H, Bortlik K, Rentsch D, Schellenberg M, Matile Ph (1989) Catabolism of chlorophyll in vivo: significance of polar chlorophyll catabolites in a non-yellowing senescence mutant of Festuca pratensis Huds. New Phytol 111:3–8

Vicentini F, Hörtensteiner S, Schellenberg M, Thomas H, Matile Ph (1995) Chlorophyll breakdown in senescent leaves: identification of the biochemical lesion in a stay-green genotype of Festuca pratensis Huds. New Phytol 129:247–252