Properties of Chlorophyllase from *Capsicum annuum* L. Fruits

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The *in vitro* properties of semi-purified chlorophyllase (chlorophyll-chlorophyllido hydrolase, EC 3.1.1.14) from *Capsicum annuum* fruits have been studied. The enzyme showed an optimum of activity at pH 8.5 and 50 °C. Substrate specificity was studied for chlorophyll (Chl) *a*, Chl *b*, pheophytin (Phe) *a* and Phe *b*, with K_m values of 10.70, 4.04, 2.67 and 6.37 μM respectively. Substrate inhibition was found for Phe *b* at concentrations higher than 5 μM. Chlorophyllase action on Chl *a*' and Chl *b*' was also studied but no hydrolysis was observed, suggesting that the mechanism of action depends on the configuration at C-13^2 in the chlorophyll molecule, with the enzyme acting only on compounds with R13^2 stereochemistry. The effect of various metals (Mg^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}) was also investigated, and a general inhibitory effect was found, this being more marked for Hg^{2+} and Fe^{2+}. Functional groups such as -SH and -S-S- seemed to participate in the formation of the enzyme-substrate complex. Chelating ion and the carbonyl group at C3 appeared to be important in substrate recognition by the enzyme. The method for measuring Chlase activity, including HPLC separation of substrate and product, has been optimized.

Introduction

Chlorophyllase (Chlase) (Chlorophyll-chlorophyllide hydrolase, EC 3.1.1.14), an intrinsic membrane-bound enzyme located in photosynthetic systems of higher plants and algae (Fernández-López *et al.,* 1992; Shioi and Sasa, 1986; McFeeters *et al.,* 1971), has been extensively studied since its discovery (Willstätter and Stoll, 1913). For long time there has been a controversy about the main function of the enzyme. Today it is widely accepted that Chlase catalyses the *in vivo* hydrolysis of chlorophylls (Chls) to chlorophyllide (Chlide) and phytol during degreening in leaf senescence and fruit ripening, and during normal turnover of chlorophylls. Other studies have shown that *in vivo* Chlase also may add the alcohol phytol to the C-13 in Chlide at the final step of Chl biosynthesis (Fiedor *et al.,* 1992). However, an enzyme, named Chl synthase, was reported to be responsible for the final step of Chl synthesis *in vivo* (López *et al.,* 1996; Rüdiger *et al.,* 1980). The recent cloning of genes for Chlase isozymes from different sources (*Chenopodium*, *Arabidopsis* and *Citrus*) (Jacob-Wilk *et al*., 1999; Tsuchiya *et al*., 1999) will help to elucidate the physiological roles of Chlase and other chlorophyll catabolic enzymes (Takamiya *et al*., 2000). Brown *et al.* (1991) have classified the reactions involved in Chl degradation into two groups, type I and type II, with the reaction catalyzed by Chlase allocated to the first group. Type I reactions are those leading to loss of Mg or phytol, oxidation, modification of peripheral substituents, and opening of the isocyclic ring V, whereas type II reactions are those leading to cleavage and opening of the porphyrin macrocycle. The order of the type I reactions is not clear – it is not well understood whether the excision of phytol is preceded or not by Mg loss. Recent experiments using ^{14}C-labeled Chls and a crude extract of *Chenopodium album* have suggested that phytol is split before Mg loss (Shioi *et al.,* 1991).

Chlase exhibits a high specificity towards the substrate, and recently it has also been found to be stereospecific, the carboxymethyl in the C13^2 chiral group playing a very important role in the interaction between substrate and enzyme (Fiedor *et al.,* 1992). Action of the enzyme on substrates

Abbreviations: β-ME, β-mercaptoethanol; Chl, chlorophyll; Chlide, chlorophyllide; DTT, dithiothreitol; IAE, iodoacetamide; MMTS, methylmethanethiosulfonate; NEM, N-ethylmaleimide; *p*-HMB, *p*-hydro×ymercuribenzoate; Phe, pheophytin; Pheide, pheophorbide.

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such as Chl *a*, Chl *b*, Phe *a* and Phe *b* have been studied with enzyme preparations from different sources (Schoch and Ihl, 1998; Fiedor *et al.*, 1992; Tanaka *et al.*, 1982). The enzyme shows no dependence on the metal ion (Vezitskii and Sherbakov, 1987), whereas Chl synthase exhibits a high dependence on the metal chelated by the macrocycle (Helfrich and Rüdiger, 1992; Benz and Rüdiger, 1981).

In the present study, Chlase from *C. annuum* fruits has been partially purified, and its properties, including substrate specificity, have been studied. *C. annuum* is a carotenogenic fruit, and therefore during ripening a massive *de novo* synthesis of carotenoid pigments takes place, which is accompanied by a sharp decrease of Chls as a consequence of the degeneration of chloroplasts in chromoplasts. During this process the role of Chlase seems to be important, and its activity is increased and/or activated by the ripening process, so that it is perhaps a factor modulating the *de novo* biosynthesis of carotenoid pigments. The level of Chlase activity is affected by internal and external factors (Drazkiewicz, 1994). The increase in Chlase activity has been related with nutritional deficiencies, and physiological status of the plant such as senescent and maturation, when deorganization of the chloroplast helps to put the latent enzyme and the substrates in contact (Terpstra and Lambers, 1983). Therefore a preliminary characterization of the enzyme, and optimization of the activity assay, are needed before attempting to study the role of Chlase during ripening of *Capsicum* fruits.

Materials and Methods

Plant material

Green fresh fruits of peppers (*C. annuum* L.) cv. Agridulce were used for the present study. Plants were cultivated in soil at "La Vera" (Cáceres, Spain) by local growers, and harvested when the green fruits were fully developed.

Extraction and partial purification of the enzyme

The method proposed by Terpstra and Lambers (1983) was used with some modifications. One hundred grams of fruits, cleared of seeds, were chopped and extracted with 100 ml of acetone at −20 °C by using a homogenizer Ultraturrax T25 (Janke Kunkel IKa-Labortechnik), and left for 15 min at −20 °C. Supernatant was collected by vacuum filtration through Whatman N°1 filter paper, and the filtrate extracted again with 100 ml of acetone. This operation was repeated until no color was observed in the extract (5 times). Finally, a white precipitate (acetone powder) was obtained, dried at room temperature, and weighed. Acetone powder (ca. 10 g) was extracted with 500 ml of 5 mM Na phosphate buffer (pH 7.0), containing 50 mM KCl and 0.24% (w/v) Triton X-100. The suspension was stirred for 1 h at room temperature and then filtered through four layers of cheesecloth. The filtrate was centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatant was semipurified by fractionation with $(NH_4)_2SO_4$ (30–60% w/v), and desalted through a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with extracting buffer. The resulting extract (35 ml) is the partially purified extract used in this study.

Substrate and standards preparation

Chl *a* and Chl *b* were isolated from fresh spinach leaves by means of acetone extraction and transfer to diethyl ether. Both pigments were separated by TLC on silicagel GF_{254} using the developing mixture petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1 v/v). Bands for Chl *a* and Chl *b* were scraped off at R_f 0.51 and 0.45, respectively. Phe *a* and Phe *b* were prepared from pure Chl solutions in diethyl ether, by acidification with a few drops of HCl 10% (v/v). The resulting Phes were transferred to diethyl ether, evaporated and dissolved in acetone. Purity of the products was checked by TLC under the same conditions used for Chls. Epimerization of both Chl *a* and *b* was achieved as proposed by Fiedor *et al.* (1992). The pigment (0.5 μmol) was dissolved in 25 ml of triethylamine and stirred in the dark during 5 h at room temperature. Chl *a*' and Chl *b*' were separated and isolated from the reaction mixture by HPLC (Mínguez-Mosquera *et al.*, 1991).

Activity assay

To characterize the enzyme, the following assay procedure was optimized. The reaction mixture contained 0.1 μmol of substrate (Chl *a*) dissolved

in acetone (0.1 ml), 100 mM Tris (tris[hydroxymethyl]aminomethane)-HCl buffer at pH 8.5, and the purified chlorophyllase extract in a ratio 1:5:5 (v/v/v; total volume 1.1 ml). The reaction was carried out using Eppendorf® microfuge tubes in a thermostatic bath at 50 °C in the dark during 1 h with continuous shaking. The reaction was stopped by freezing at −20 °C until analysis. For HPLC analysis an aliquot of the sample (250 µl) was diluted with THF in a relation 1:1 and centrifuged at 13,000 × *g* for 5 min, and 20 µl injected in the chromatograph. HPLC analyses were carried out using a Waters 600E quaternary pump equipped with a photodiode array detector (PDA 996, Waters) and controlled with a Millennium data acquisition station. Chromatographic separation was carried out using the method of Mínguez-Mosquera *et al.* (1991) but with a few modifications in the gradient profile. This method allows the separation of both the remaining substrate and the reaction product Chlide. A reversed phase column (Spherisorb ODS2, 5 µm, 25 cm × 0.46 cm), and a binary solvent system consisted of A (water/tetrabutylammonium 0.5 M, ammonium acetate 1 M/methanol, 1:1:8 v/v) and B (acetone/methanol, 1:1 v/v) were used. The gradient profile was optimized as follows: program started with a linear gradient from 75% to 40% of A in 5 min, continuing by a convex gradient (curve 1 in the pump Waters 600E) to 10% of A in 3 min, and followed by a linear step until 0% A (100% B) in 4 min, and finally returned with a convex gradient to the initial conditions in 3 min. In the case of using Phes as substrates, the gradient started at 90% of A to allow the separation and detection of the Pheides polar products. Flow rate was 2.0 ml·min^{-1}. Detection was performed using a detection wavelength of 432 nm for Chl *a*, 466 nm for Chl *b*, 410 nm for Phe *a* and 434 nm for Phe *b*. Quantification was achieved by external calibration curves with pure standard solutions.

Metal ions and functional group reagents were introduced in the Tris-HCl buffer at a concentration 10 µM and 1 mM respectively.

Protein determination

Protein content of different fractions was measured according to Bradford (1976). Bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, MO) was used as standard.

Statistical analysis

All experiments were carried out in triplicate. The data were subjected to analysis of variance (ANOVA) and Duncan's method. Trends were considered significant when means of compared data differed at $P<0.05$.

Results and Discussion

Isolation and partial purification

Chlorophyllase is associated with chloroplast membranes which requires disruption and disintegration of the membrane structure to extract the enzyme. Our initial approach involved the direct extraction of the enzyme from fresh fruit using Na phosphate buffer at pH 7.0 and containing 0.1% (w/v) Triton X-100. Chlorophyllase activity was observed, however, the extract was greenish, indicating the presence of chlorophyll which can disturb the results of measured activity.

Therefore protein precipitation with acetone at −20 °C was chosen, five successive extractions being necessary to get a white protein precipitate. Approximately 10 grams of fresh fruit yielded 1 gram of protein precipitate which was then extracted with 5 mM Na phosphate buffer (pH 7.0), containing 50 mM KCl and 0.24% (w/v) Triton X-100, in a ratio of 1:50 (w/v) for 1 hour at room temperature and with continuous stirring. After filtration and centrifugation, a crude extract was obtained. Enzymatic activity was maximal after 40 min, but 60 min was selected for the routine extractions.

A partial purification was achieved by fractionating the crude extract by precipitation with ammonium sulfate. Chlorophyllase enzyme was present in the protein fraction comprising 30 to 60% saturation. The precipitate was redissolved in a minimum volume of extracting buffer and desalted using a PD-10 column previously equilibrated with extracting buffer; the resulting product was the semi-purified extract.

Effect of pH and temperature

A broad peak of maximum chlorophyllase activity was found at pH 8.5. Decreases of 35 and 60% in activity were detected at pH 7.0 and 11.5 respectively. Similar results have been obtained by Tamai *et al.* (1979) with semi-purified chlorophyllase

from *Chlorella*, and by Khalyfa *et al.* (1993) with chlorophyllase from *Phaeodactylum tricornutum*. During ripening chloroplasts are transformed into chromoplasts, which leads to deorganization of the thylakoid membranes, and may provide enzyme access to Chl molecules. Moreover, the fact that the intra-thylakoid space (as a consequence of the photosynthetic activity) has basic pH conditions, close to the optimum chlorophyllase pH, maybe important for the *in vivo* action of the enzyme. Activity was measured in the temperature range 30–70 °C, and exhibited a maximum at 50 °C. These results were in agreement with those found in *Olea europaea* (Mínguez-Mosquera *et al.*, 1994) and *Canola* (Levadoux *et al.*, 1987). Denaturation kinetics were determined at four temperatures (40, 50, 60 and 70 °C). The enzyme remained stable at 40 °C for 1 hour, whereas at 50 °C it was slightly degraded after 40 min, and more rapidly at higher temperatures. Storage temperature conditions at −20, 4 and 25 °C were also assayed. At 25 °C, the enzyme lost 50% of activity in 6 days, whereas at 4 °C it took 18 days to reach the same value. At −20 °C, its activity remained unchanged for at least one year.

Substrate specificity

Substrate specificity has been studied for Chl *a* and *b*, as well as for Phe *a* and *b*, measuring in each case the amount of product formed, Chlides and Pheides respectively. Incubations were carried out with increasing concentrations of substrate in the range 2 to 60 μM. Fig. 1 shows the kinetic curves obtained, in each case, all four substrates, exhibited a typical Michaelis-Menten kinetic curve. However, substrate inhibition was observed for Phe *b* at concentration higher than 5 μM. Lineweaver-Burk plots (Fig. 1) were used to calculate kinetic parameters, K_m and V_{max}. The obtained values were similar to those found by other authors using other plants and algae; for instance, K_m is 10 μM for Chl *a* in tea leaves (Kuroki *et al.*, 1981), 12 μM in rye seedlings (Tanaka *et al.*, 1982), and 7.0 μM in *Chlorella regularis* (Nishiyama *et al.*, 1994).

The K_m value indicates that the enzyme has a higher affinity for Chl *b* than for Chl *a*; however, V_{max} was higher for Chl *a*, indicating a faster transformation of this substrate by the enzyme, which is in agreement with previous studies on olive (*Olea europaea*) (Mínguez-Mosquera *et al.*, 1994) and 'satsuma' mandarin (Shimokawa, 1979). Nevertheless, the enzyme showed its strongest affinity for Phe *a* (K_m= 2.67 μM), although with a V_{max} lower than for Chl *a*. These results suggest the importance of some structural features for the recognition of the substrate by the enzyme. A higher K_m value for Mg-free substrates (i.e. pheophytins) suggested an important role of the chelated ion, which is less specific and therefore yields a lower V_{max}. Structural differences between *a* and *b* chlorophylls at the C3 carbon (-CH3 and -CHO, respectively) may also explain the higher apparent affinity for Chl *b*, which indicates an involvement of the carbonyl group in substrate recognition. The substrate inhibition shown by the enzyme for pheophytin *b* might also corroborates this point, since this pigment has both structural characteristics (Mg-free and carbonyl at C3) which makes this substrate less specific.

During the course of the present study, an interesting phenomenon was observed: the standard solutions of Chl *a* and *b* always contained small amounts of their $C13^2$ epimers, Chl *a*' and *b*' respectively. When these solutions were used for the chlorophyllase assay, a preferential action on the non-epimeric forms was found. To investigate this observation standard solutions of Chl *a*' and *b*' were prepared and incubated with chlorophyllase at various concentrations. No de-esterification was observed with the $C13^2$ epimeric forms, which is in accordance with previous reports (Nishiyama *et al.*, 1994; Fiedor *et al.*, 1992). They concluded that chlorophyllase recognizes the $C13^2$ stereochemistry, hydrolyzing only the *a* and *b* forms whose stereochemistry is 13^2R, that is, oriented in opposite direction to the propionic acid residue on C17. Fiedor *et al.* (1992) suggested that $C13^2$ could be involved in the formation of the enzyme-substrate complex with an enzyme similar to the normal chlorophyllase being necessary for the $C13^2$ epimeric forms. Such enzyme might be a modified chlorophyllase.

Effects of metal ions

Extracts were incubated for 24 h at 4 °C in the presence of various metal ions (Mg^{2+}, Mn^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+}) at 10 μM of con-

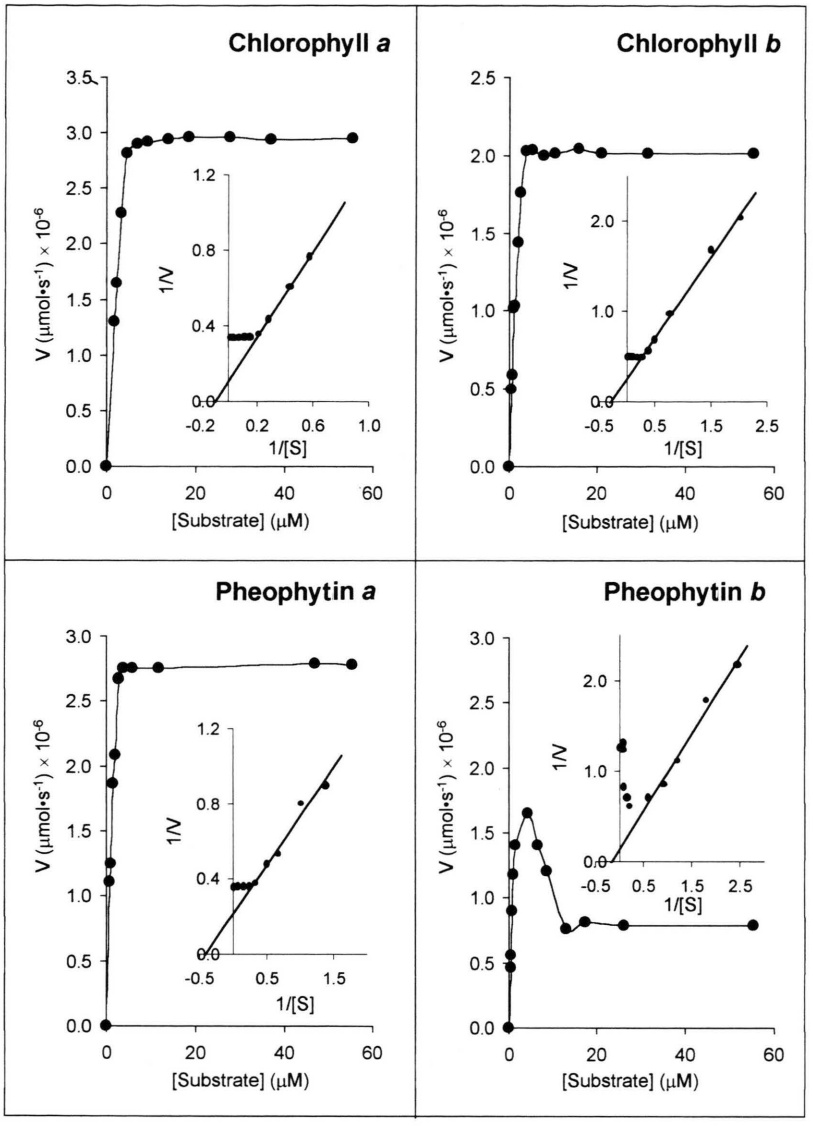

Fig. 1. Enzyme kinetic curves and Lineweaver-Burk transformation plots for chlorophyllase activity with Chl *a*, Chl *b*, Phe *a* and Phe *b*. K_m (μM) and V_{max} (x 10^6 μmol s^{-1}) were 10.70 ± 0.53 and 9.33 ± 0.38 for Chl *a*, 4.04 ± 0.17 and 4.48 ± 0.21for Chl *b*, 2.67 ± 0.06 and 5.01 ± 0.18 for Phe *a*, and 6.37 ± 0.25 and 7.49 ± 0.27 for Phe *b*, respectively.

centration. In parallel, an enzyme control solution without any metal ion was incubated under the same conditions, and the activity was assayed as described before. The results are documented in Table I. All ions inhibited, although Mg and Fe(III) only slightly, which are both closely related to the Chl biosynthetic pathway, and therefore they may be present in the *in vivo* environment of chlorophyllase. Metals such as Cu, Zn, and Co may react with sulfhydryl groups of the amino acid residue cysteine, indicating the need for such groups in the action mechanism of the enzyme. This was observed again with Hg and Fe(II), which are highly reactive with these functional groups, leading to a high degree of inhibition.

Table I. Metal effect on chlorophyllase activity. Metal concentration was 10 μM in 100 mM Tris-HCl pH 8.5. Incubation time was 24 h prior to enzyme activity assay.

Metal ion	Inactivation (%)
Control	0
Mg^{2+}	12 ± 2[a]
Mn^{2+}	67 ± 4
Hg^{2+}	85 ± 5
Cu^{2+}	55 ± 4
Zn^{2+}	60 ± 4
Co^{2+}	56 ± 3
Fe^{2+}	89 ± 5
Fe^{3+}	13 ± 2

[a] Mean and standard deviation of three replicates.

Functional groups involved

Little effect of iodoacetamide (IAE), N-ethylmaleimide (NEM), and methyl methanethiosulfonate (MMTS) was found, but *p*-hydroxymercuribenzoate (*p*-HMB) almost totally inhibited activity (Table II). These results not only indicate the existence of sulfhydryl groups in the enzyme, but also suggest the involvement of some of them at the active sites, which are not easily available to weak reagents. We can deduce that the active site may contain at least one sulfhydryl group participating in the formation of the substrate-enzyme complex, which was inhibited by *p*-HMB. The results agree with those of Kuroki *et al.* (1981) for chlorophyllase from tea leaves, although McFeeters *et al.* (1971) concluded the absence of these functional groups in chlorophyllase from *Ailanthus altissima*.

Table II. Involvement of functional groups in chlorophyllase activity. Reagent concentration was 1 mM in 100 mM Tris-HCl, pH 8.5. The enzyme was incubated in presence of the reagent for 24 h at 4 °C.

Reagent	Activity (%)
Control	100
IAE	93 ± 3[a]
NEM	87 ± 4
p-HMB	17 ± 2
MMTS	97 ± 2
DTT	113 ± 8
β-ME	115 ± 6

[a] Mean and standard deviation of three replicates.

The involvement of -S-S- bonds was investigated using dithiothreitol (DTT) and β-mercaptoethanol (β-ME) as reducing agents. The results obtained (Table II) indicate an activating effect of the enzyme when the -S-S- groups are reduced to -SH. As mentioned before the blocking of -SH groups leads to an inhibitory effect. In this case, reduction of -S-S- bonds may modify the active conformation, increasing the accessibility of the substrate to the active site.

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