Substrate Specificity of Chlorophyllase from Different Plants

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The activity of chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) extracted from six different species was compared with enzyme extracted from leaves of Tree of Heaven. The chlorophyllase activity from Swiss chard was similar to the Tree of Heaven enzyme, all the others were less active or inactive. We tested the substrate specificity with bacteriochlorophyll *a*, chlorophylls *a* and *b*, pheophytins *a* and *b* and also the synthetic pigments Zn pheophytins *a* and *b* and Zn pyropheophytin *a*. The natural pigments were the best substrates, but the Zn derivatives were also hydrolysed, except Zn pyropheophytin *a* which was accepted only by the enzyme extracted from the leaves of Tree of Heaven.

Introduction

Chlorophyllase (chlorophyll-chlorophyllido hydrolase, EC 3.1.1.14) is an intrinsic membrane enzyme present in most photosynthetic membranes of higher plants (Schellenberg and Matile, 1995). Since the discovery of chlorophyllase (Chlase) by Willstätter and Stoll (1913), the function of the enzyme *in vivo* remained controversial. Evidence has been found that Chlase intervenes in the synthesis of chlorophyll (Chl) (Minguez-Mosquera and Gallardo-Guerrero, 1996; Fiedor *et al.,*1992; Holden, 1976). Other authors state that the enzyme is involved in an early stage of the Chl catabolism in senescing leaves (Hörtensteiner *et al.*, 1995; Vicentini *et al.,* 1995) but also under not natural conditions in Chl degradation in food processing (Simpson *et al.,* 1976; Heaton and Marangoni, 1996). Many investigations have been directed at the physiological properties of the enzyme using crude extracts or partially purified preparations (Khalyfa *et al.* 1993; Shioi *et al.*, 1980; McFeeters *el al.*, 1971). The enzyme was extracted from leaves (Levadoux *et al.,* 1987; Bacon and Holden, 1970), fruits of higher plants (Trebitsh *et al.*, 1993; Minguez-Mosquera *et al.,* 1993; Hirschfeld and Goldschmidt, 1983) or algae (Shioi *et al.*, 1980; Terpstra, 1981). Most of the reports focus on the purification and characterization of the enzyme, and only few publications deal with the problem of substrate specificity. McFeeters (1975) decribes the substrate specificity of partially purified Chlase from Tree of Heaven with different alcohols of the propionic esters of Chl *a* and *b* and the corresponding Mg-free derivatives. The influence of some peripheral substituents and the stereospecificity at C-13[2] was investigated by Fiedor *et al.* (1992), using crude enzyme preparation from *Melia azedarach* L. Zn derivatives can substitute for the natural pigments in photosynthesis units of bacteria (Wakao *et al.* 1996). These pigments were also found active in the stabilization of Chl *a*-binding apoproteins in barley (Eichacker *et al.*, 1996) and several enzymatic reactions of Chl biosynthesis (Scheumann *et al.*, 1996; Schoch *et al.*, 1995; Helfrich and Rüdiger, 1992; Griffiths, 1980). The Zn derivatives can also be produced in food processing, being responsible for the remaining green color during thermal processing of vegetables (LaBorde and von Elbe, 1994; Tonucci and von Elbe, 1992).

Comparisons among preparations made in different laboratories are difficult because of differences in the methods of assay and of reporting results. Here we report a comparative study of the activity of Chlase extracted from three different plants, with different Chl derivatives: the naturally occuring Mg-complexed Chl *a, b*, Bchl *a* and the

Abbreviations: Bchl, bacteriochlorophyll; Chl, chlorophyll; Chlase, chlorophyllase; Phe, pheophytin; Znphe, zinc pheophytin; Znpyrophe, zinc pyropheophytin.

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Mg-free compounds, with the Zn-complexed derivatives. The other goal of this study was to compare under identical experimental conditions the enzyme activity of different vegetables grown at the same location and same vegetation period, with enzyme preparation from leaves of Tree of Heaven.

Material and Methods

Pigments

Chl *a* and *b* were isolated from spinach leaves and purified according to Iriyama *et al.*, (1979). Bacteriochlorophyll *a* (Bchl a) was supplied by H. Scheer. Pheophytin (Phe) *a* and *b* were obtained by acidification of the respective Mg compound. Pyropheophytin *a* was prepared from Phe *a* according to Pennington *et al.* (1964). Zinc was inserted into the corresponding Phe according to Helfrich *et al.* (1994).

Plant material

The leaves of Tree of Heaven (*Ailanthus altissima*) were harvested in the Botanical Garden in Munich (Germany). The horticultural products were grown in Temuco (Chile, 39° S latitude), and the following parts used as enzyme source: leaves from Swiss chard (*Beta vulgaris* L. cv. cicla L.) and spinach (*Spinacia oleracea* L.), immature flower from Maipo-ecotype globe artichoke (*Cynara scolymus* L.), and immature seeds of peas (*Pisum sativum* L., cv. Farmer and Bolero). The shoots of the green asparagus (*Asparagus officinalis* L.) were divided into the tip (upper 2 cm) and stem (lower 3–4 cm). The veins of the leaves, the outer bracts from the artichoke flowers, the pods of the peas, the outer layer of the shoots were removed. The remaining plant material was frozen.

Chlorophyllase preparation

The frozen material was blended in a mixer with cold acetone (30 g/l) and washed immediately three times with cold acetone. The pigmented residue was washed with different acetone/water mixtures (80, 70, 55, 90, finally 100% acetone) until the powder was colourless. Between each washing, vacuum filtration was used. Finally the powder was dried at ambient temperature overnight, and kept refrigerated. 300 mg of the acetone powder (150 mg for Swiss chard and Tree of Heaven) in 10 ml 100 mM phosphate buffer (pH 7.5) containing 0.6% (v/v) Triton X-100 and 30 mg Na ascorbate, was stirred overnight in the cold room (8 °C), centrifuged for 30 min at 12,000×*g*, and the supernatant containing the solubilized enzyme was collected. Protein content of the enzymes was determined following the method of Bradford (1976), using the Bio-Rad assay mixture (Bio-Rad Laboratories, Munich, FRG) and bovine serum albumin as a standard.

Chlorophyllase assay

The crude extract of Chlase, containing about 10^{-5} M substrate and 0.1% pyridine (to avoid pheophytinization) was incubated in darkness at 30 °C for the time required. The reaction was stopped adding acetone up to 80%. The amount of pigment was calculated from the respective absorption spectra, with the molar absorption coefficients at the red maxima: 81.0×10^3 $M^{-1}cm^{-1}$ for Chl *a*, 47.6×10^3 $M^{-1}cm^{-1}$ for Chl *b*, 49.2×10^3 $M^{-1}cm^{-1}$ for Phe a, 31.6×10^3 $M^{-1}cm^{-1}$ for Phe *b*, 70.9×10^3 $M^{-1}cm^{-1}$ for Bchl *a* (Scheer, 1988), 77.3×10^3 $M^{-1}cm^{-1}$ for zinc pheophytin (Znphe) *a* and zinc pyropheophytin (Znpyrophe) *a* (Helfrich and Rüdiger, 1992), 51.3×10^3 $M^{-1}cm^{-1}$ for Znphe *b*. The esterified pigments were extracted into hexane as described by Helfrich *et al.* (1994) and the amount of esterified pigment was calculated from the absorption maxima as before with the molar absorption coefficients: 90.2×10^3 $M^{-1}cm^{-1}$ for Chl *a*, 56.2×10^3 $M^{-1}cm^{-1}$ for Chl *b* and 91.0×10^3 $M^{-1}cm^{-1}$ for Bchl *a* (Scheer, 1988), 90.3×10^3 $M^{-1}cm^{-1}$ for Znphe *a*, 60.2×10^3 M^{-1} cm^{-1} for Znphe *b* (Jones *et al.*, 1977) and 80.0×10^3 $M^{-1}cm^{-1}$ for Znpyrophe *a* (Pennington *et al.*, 1964). Control experiments were done with enzymes heated at 100 °C for 5 minutes. All values were corrected by the control value.

Results and Discussion

Comparison of chlorophyllase activity obtained from leaves of Tree of Heaven, Swiss chard and of the tip of shoots from asparagus

a. Activity with the natural substrate Chl *a*

Figure 1 shows the time-dependent hydrolysis of Chl *a* with three enzyme preparations. The rate of reaction of the extracts from Swiss chard and Tree of Heaven are similar although the concentration of the protein of the later enzyme preparation was about three times of that from Swiss chard (3.5 g/l protein in Tree of Heaven and asparagus, whereas 1.2 g/l protein in Swiss chard). After 10 min about 80% of the Chl *a* was hydrolysed. The reaction then slowed down drastically, after 60 min 10 to 15% of the original pigment remained. The situation was different for the enzyme extracted from asparagus, where the initial fast phase was barely discernible, and the overall rate of reaction was much slower. Nonetheless, after 60 min the amount of remaining esterified pigment in the three enzyme preparations is comparable. From these results it is not possible to decide if the difference in activity is due to different amounts of enzyme in the crude extracts or if the kinetic properties are different.

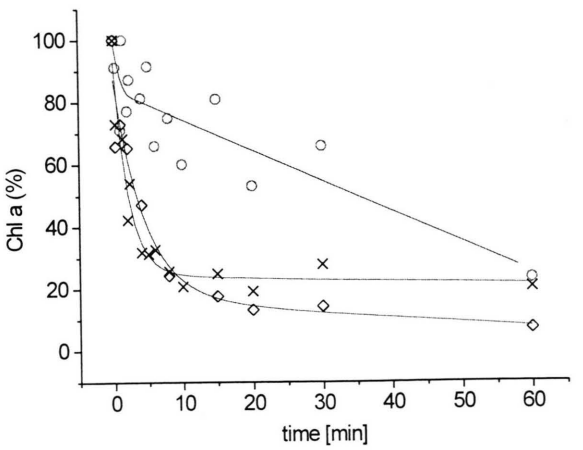

Fig. 1. Progress of Chl *a* hydrolysis by enzyme preparations from leaves of Tree of Heaven (x), Swiss chard (◊) and tips (upper 2 cm) of green asparagus (o). The curves were fitted by biexponential fit.

b. Chlorophyllase reaction with different substrates

As mentioned before, only few papers deal with the substrate specificity of Chlase. Fiedor *et al.* (1992) used crude extracts from leaves of China tree (*Melia Azedarach* L.) to hydrolyse the natural substrates Chl *a*, *b*, and Bchl *a*. Mc Feeters (1975) and Minguez-Mosquera *et al.* (1994) reported the hydrolysis of the Mg-free pigments. In contrast to this finding, the enzymes catalyzing the last steps of biosynthesis of Chl do not accept the metal-free pigments as substrate but they do accept the zinc derivatives (Griffiths, 1980, Schoch *et* al, 1995, Scheumann *et al.* 1996). Eichacker *et al.* (1996) showed, that Chl *a*-binding apoproteins can also be stabilized by Znphe *a* produced from zinc pheophorbide *a* during esterification with the enzyme chlorophyll synthase *in vitro*. To compare the substrate specificity of this enzyme with Chlase, which catalyzes the hydrolysis of pigments, we tested the Chlase reaction with Bchl *a*, Chl *a* and *b*, the Mg-free derivatives pheophytin *a* and *b*, and the zinc derivatives Znphe *a* and *b*. McFeeters (1975) found that the removal of the C-13^2 carboxymethyl group has only a minor effect on substrate binding to the enzyme; therefore, we tested also Znpyrophe *a*.

The very fast hydrolysis of Chl *a* with extracts from leaves of Tree of Heaven and Swiss chard, compared with the slow reaction of the asparagus enzyme (Fig. 1), prompted us to use reaction times of 15 and 30 min for the different substrates. Of the three sources of enzyme, the leaves from Tree of Heaven are apparently the best for all substrates tested (Table I). Chl *a* and Bchl *a* are the best substrates, followed by Chl *b*, Znphe *a* and *b*. The hydrolysis of Znpyrophe *a*, Phe *a* and *b* proceeds much slower, although after 30 min between 50 and 60% of the pigments are saponified. The central ion of the chlorin macrocycle influences the reactivity of the enzyme, being best Mg, followed by Zn and finally hydrogen (e.g. Chl *a*, Znphe *a* and Phe *a*). Both, substitution of a formyl group for a methyl group at position 7 or removal of the carboxymethyl group in the isocyclic ring E also decreases the rate of Chlase reaction. Michalski *et al.* (1987) found that the apparent inhibition in Chlase catalyzed reactions of Chls in the presence of Triton X-100 is likely to be due to competitive transesterification reactions in which the de-

Table I. Comparison of the reaction of enzyme extracts from Tree of Heaven, Swiss chard and the tip of shoots of asparagus (u.p.) with different substrates. The values are expressed as percentage of remaining esterified pigment after 15 or 30 minutes reaction time at 30 °C. They were calculated from two to five individual experiments with standard deviation except for the marked values (*) which are the values of a single experiment.

Substrates	Tree of Heaven		Swiss chard		Asparagus u.p.	
	15 [min]	30 [min]	15 [min]	30 [min]	15 [min]	30 [min]
Chl *a*	17.6 ± 1.1	14.3 ± 1.8	24.8 ± 4.7	27.8 ± 12.6	80.8 ± 9.1	65.7 ± 14.1
Chl *b*	35.6 ± 12.2	22.5 ± 13.2	26.1 ± 11.6	14.9 ± 0.4	93.9 ± 7.9	69.7 ± 2.5
Bchl *a*	17.1 ± 0.8	19.7 ± 3.4	36.8 ± 16.0	23.9 ± 9.7	91.8 ± 3.3	77.2 ± 3.7
Znphe *a*	32.0 ± 17.2	25.8 ± 13.6	30.0 ± 10.5	20.5 ± 4.6	85.7 ± 7.9	66.9 ± 2.5
Znphe *b*	43.9 ± 9.5	32.7 ± 5.0	45.1 ± 3.6	41.7 ± 5.2	78.4 ± 1.0	63.0 ± 5.1
Znpyrophe *a*	66.9 ± 11.9	38.6 ± 4.7	103.3 ± 0.9	94.0 ± 0.8	89.9 ± 0.8	80.8 ± 0.8
Phe *a*	68.2 ± 0.4	35.7*	101.6 ± 9.4	63.2 ± 1.7	91.9*	66.9*
Phe *b*	68.1 ±12.0	47.5 ± 13.0	97.3*	67.8 ± 0.6	97.8*	72.5*

tergent is the substrate, in particular for Chl derivatives lacking the carboxymethyl group in ring V.

On the other hand, comparison of the three enzymes shows that not only the substrates influence the reactivity but also the source of the enzyme. Znpyrophe *a* is saponified by the enzyme extracted from Tree of Heaven whereas it is not accepted at all by the Swiss chard enzyme. With none of the substrates tested the Chlase activity of the extract from asparagus is comparable with the other two Chlase preparations.

Comparison of the activity of chlorophyllase extracted from different plants

The substrate specificity data shows that the enzyme has very specific requirements. This has significant implications for studies defining the role of the enzyme in Chl metabolism. If Chlase is an essential enzyme in Chl synthesis or degradation, very similar substrate specificities for the different plants are to be expected. A comparison of Chlase activity in a survey of five higher plant species showed that tobacco has the lowest and China tree the highest activity (Amir-Shapira *et al.*, 1987). Another study by Levadoux *et al.* (1987) compared the Chlase activity of sugar beet leaves with alfalfa and ryegrass. Pennington *et al.* (1964) describe differences in the activity of Chlase extracted from the same plants but grown at different locations.

Our approach was to measure Chlase activity of tissues from different green vegetables grown during the same year and at the same location (Temuco, Chile), and to compare them with the activity of the Tree of Heaven enzyme. The substrates used were Chl *a* and two synthetic derivatives Znphe *a* and *b* (Table II). The vegetable Swiss chard was chosen due to the high Chlase activity of sugar beet, another *Beta vulgaris* plant, found by Levadoux et al (1987) and Bacon and Holden (1970). The other horticultures were the leaves of spinach, the immature flowers of artichokes, different varieties of pea seeds used as vegetable, and different parts of a horticulture, the tip and the stem of green asparagus shoots.

Table II. Comparison of Chlase activity extracted from different plants. The activity is expressed in percentage of saponified pigment after 30 min reaction time. The shoots of the green asparagus were divided into the tip (upper 2 cm, u.p.) and stem (lower 3–4 cm, l.p.). The values and standard deviations from the first three enzyme extracts were calculated from three to five, the other from two individual experiments.

Plants	Chl *a*	Znphe *a*	Znphe *b*
Tree of Heaven	87 ± 2.4	78 ± 6.3	66 ± 6.2
Swiss chard	81 ± 1.4	79 ± 3.8	58 ± 6.3
Asparagus u.p.	40 ± 17.9	26 ± 10.7	25 ± 9.0
Asparagus l.p.	0	9 ± 6.7	<5
Spinach	42 ± 7.4	7 ± 2.8	<5
Pea Bolero var.	28 ± 0.4	0	<5
Pea Farmer var.	49 ± 0.9	<5	0
Artichoke	0	<5	6 ± 2.7

The Chlase prepared from leaves of Tree of Heaven and from Swiss chard is the most active. Chlase extracted from spinach showed activity for the reaction with Chl *a*, but almost no activity with the zinc compounds. Extracts from the stem of asparagus shoot, a more mature tissue than asparagus tip, did not show any activity with Chl *a* and

Znphe *b*, but some activity with Znphe *a*. whereas the asparagus tip extracts show Chlase activity for all three substrates tested. By contrast, the enzyme from artichoke, also an immature tissue, was almost inactive for all pigments tested. It should be noted, that Chlase extracted from fresh artichokes and analysed immediately with a Chl *a/b* mixture, was active (Ihl *et al.*, in press). From the peas tested, the enzyme extracted from cv. Farmer seemed to be more active with Chl *a* than the one from cv. Bolero. With the Zn derivatives both varieties were inactive. It is interesting to note that La Borde and von Elbe (1994) found heat stable green colored Zn derivatives of Chl in thermally processed pea puree in presence of Zn ions. On the other hand, the Zn concentration was above the FDA limit of 75 ppm for human consumption and Chlase must have been inactivated during the heat processing.

The results show surprising differences in both activity and specificity of Chlase from different green tissues, and even among different varieties of the same species or different tissues of the same plant. All the experiments were performed with the crude enzyme extracts of the aceton powders prepared under identical conditions. By a brief trypsin treatment, Böger (1965) could activate Chlase extracted from Chlorella. It has to be tested, if similar treatment would have the same effect with our preparations. The metabolic relevance of these variations, which may reflect different ways of Chl degradation, clearly needs further studies.

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