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Purification and Substrate Specificity of Chlorophyllase from *Chlorella regularis*

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Chlorophyllase was purified 13300-fold from a crude butanol extract of *Chlorella regularis*. The enzyme clearly recognizes the $C13^2$ stereochemistry of chlorophyllous pigments in that it hydrolyzes the *a* and *b* (13^2R) forms but not the *a*' and *b*' (13^2S) forms. No enzyme was detected *in vivo* that specifically hydrolyzes chlorophyll *a*'.

We have lately shown that two molecules of chlorophyll (Chl) *a*', the 13^2S-isomer or epimer of Chl *a*,[1] are closely associated with P700, the primary electron donor of photosystem I in oxygenic photosynthesis.[2,3] Toward a goal of elucidating the *in vivo* function of Chl *a*', it is important to know how the $C13^2$ stereochemistry controls the properties, the intermolecular aggregation being an example,[4] of Chl analogues. Plants possess an enzyme called chlorophyllase (Chlase, EC 3.1.1.14) that hydrolyzes the $C17^3$ phytyl ester linkage of a Chl to give a water-soluble chlorophyllide (Chlid), or a pheophorbide (Pheid) from a metal-free pheophytin (Phe), probably as a first step of their degradation *in vivo*.[5-8] Since $C17^3$ and $C13^2$ are close to each other in Chl molecules, the $C13^2$ stereochemistry is expected to be a determining factor for the Chlase activity. Only one very recent report by Fiedor *et al.*[9] dealt with this particular question. Though they indeed observed five- to ten-fold faster hydrolysis of 13^2R-forms than the 13^2S counterparts for Chlase from *Melia azedarach*, an unequivocal conclusion was not reached because of possible contamination of the pigments as substrates. They used only a crude acetone extract, so that any cooperative action of plural enzymes could not be ruled out. Further, it is of much interest to examine Chlase(s) from other species. In view of these, we attempted here to purify a Chlase from a *Chlorella* species and study the substrate specificity of the purified enzyme.

Chl *a* and *b*, Phe *a* and *b*, and their epimers (denoted with a prime) of >99.5% purity were prepared as previously.[10] Chlase was extracted from *Chlorella regularis* S-50 strain (Nihon Chlorella) according to the method of Shioi and Sasa:[6] Wet cell paste 500 g was suspended in 2 L of a 10 mM phosphate buffer (pH 7.2) and homogenized by sonication (20 kHz) for 20 min. NaCl (1%) and an equal volume of butanol (-18 °C) was added to the homogenate. Further sonication (3 min) followed by 10000×*g* centrifugation (10 min) yielded an aqueous layer, which was the starting enzyme preparation (see Table 1). To this solution, $(NH_4)_2SO_4$ was added to 35% saturation and the precipitate was discarded by centrifugation at 25000×*g* for 10 min.

The supernatant (second enzyme preparation, Table 1) was submitted to four successive chromatographic steps. (1) It was first loaded on a 4 cm ϕ × 14 cm Toyopearl HW-65F hydrophobic column (Tosoh) pre-equilibrated with buffer A (50 mM phosphate, pH 7.2, plus $(NH_4)_2SO_4$ in 35% saturation). The column was washed with 1.5 L of buffer A, and developed with a linear gradient from 35 to 0% saturation of $(NH_4)_2SO_4$ in 900 mL of buffer A. Chlase-active fractions were collected and dialyzed in buffer B (1 M $(NH_4)_2SO_4$, 50 mM phosphate, pH 7.2). (2) The dialyzed solution was then applied on a 1.5 cm ϕ × 33 cm Butyl-Toyopearl 650C

hydrophobic column (Tosoh) pre-equilibrated with buffer B. The column was washed with 120 mL of buffer B, and developed with a gradient from 1 to 0 M $(NH_4)_2SO_4$ in 400 mL of buffer B. Active fractions were collected and dialyzed in buffer C (20 mM phosphate, pH 7.2). (3) The dialyzed solution was then applied on a 2.2 cm ϕ × 20 cm DEAE-Toyopearl 650S ion-exchange column (Tosoh) pre-equilibrated with buffer C. The column was washed with 100 mL of buffer C, and developed with a gradient from 0 to 0.5 M NaCl in 380 mL of buffer C. Active fractions were collected and dialyzed in buffer D (5 mM phosphate, pH 7.2). (4) The dialyzed solution was lyophilized, and the solid was dissolved in 5 mL of buffer D (20 mM phosphate, pH 7.2, 200 mM NaCl), which was finally loaded on a 2.6 cm ϕ × 67 cm Sephacryl S-200HR column (Pharmacia) pre-equilibrated with buffer D. The column was eluted with the same buffer to collect active fractions. In each chromatographic step, the Chlase activity was assayed using Chls *a* and *a*' as substrates.

The Chlase activity assay was done following McFeeters *et al.*[7] with slight modification. Ten nanomoles of Chl *a* or *a*' was dissolved in 290 μL of a 10 mM phosphate buffer (pH 7.2) containing 0.05% Triton X-100, and a 10-μL aliquot of an enzyme solution was added, followed by incubation (typically for 30 min) at 30 °C in darkness. The choice of 0.05% as the Triton X-100 concentration is based on an examination, which has not been described in the literature to date, of its effect on the Chlase activity as displayed in Fig. 1. The enzymatic reaction was stopped by adding 1.1 mL of hexane/acetone (5/3.25), then the solution was centrifuged at 10000×g for 3 min. The Chlid contained in the aqueous layer was quantitated spectrophotometrically. For comparison, the reaction was carried out also in 30 % aqueous acetone commonly used in many works.[6,8] One unit (U) of the Chlase activity is defined here as the amout of enzyme yielding 1.0 μmol of Chlid per minute.

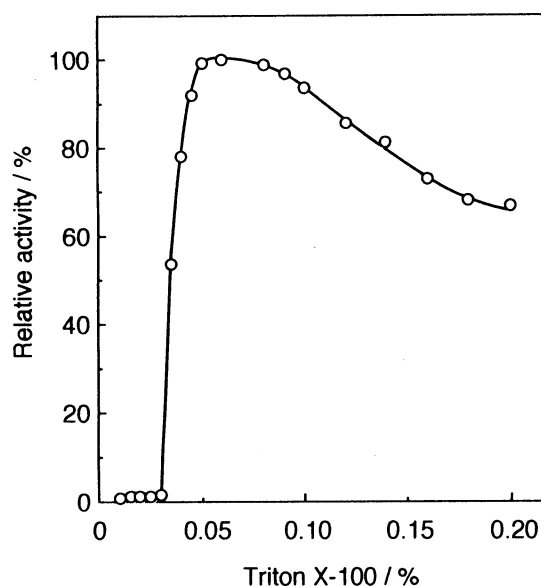

Fig. 1. Effect of Triton X-100 concentration on the relative Chlase activity of a solution collected from the Toyopearl HW-65F column.

Figure 2 illustrates the result of the third chromatographic purification stage by use of a DEAE-Toyopearl 650S column. The Chlase activity toward Chl *a* formed a single peak as a function of elution volume, indicating the absence of isozymes in the species employed. Any absorbance peak coinciding with the activity peak is buried in the dominant non-Chlase protein bands: this reflects the very low content of the enzyme in *C. regularis*. These features were common also for the first (Toyopearl HW65-F) and second (Butyl-Toyopearl 650C) chromatographic stages (data not shown).

The activity-protein peak coincidence, however, was eventually attained in the final stage with a Sephacryl S-200HR gel-filtration column, as seen in Fig. 3. The peak corresponds to a molecular mass of *ca.* 130 kDa. By SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining, the active fractions gave a single broad band at a molecular mass of *ca.* 65 kDa (data not shown), suggesting that this Chlase is a dimer of 65 kDa subunits. The minimum molecular mass is larger than 38 kDa of Chlase from *C. protothecoides*,[6] or 27-39 kDa from other algae and plants.[6] The broadness of the SDS-PAGE band may reflect the nature of Chlase, namely a membrane-bound protein[5] or a glycoprotein.[11]

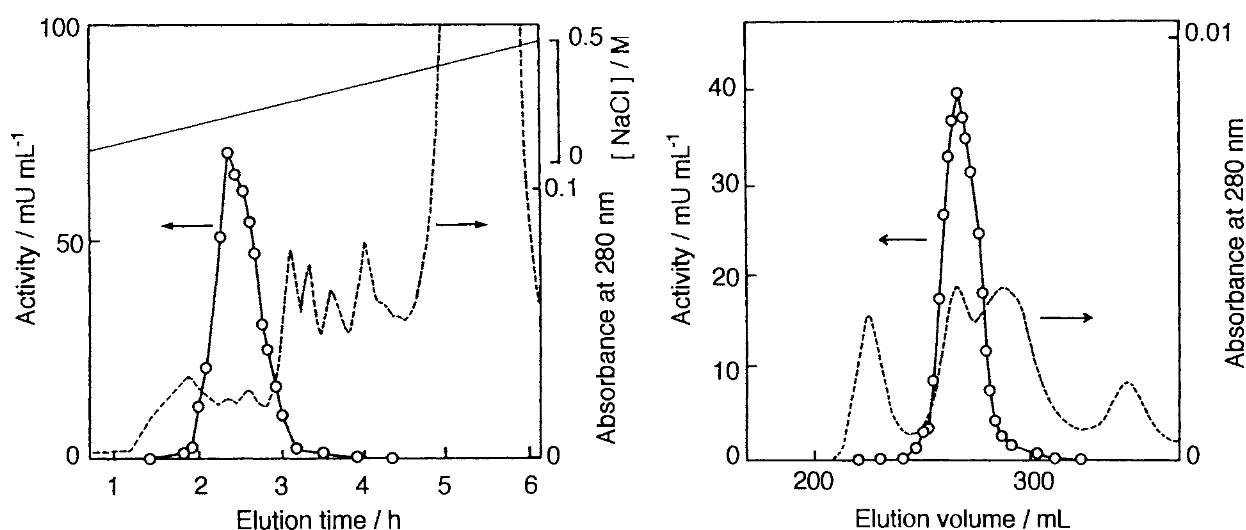

Fig. 2(left). Chlase activity toward Chl *a* (O) and 280-nm absorbance (dashed curve) for the third chromatographic purification step on the DEAE-Toyopearl 650S column.

Fig. 3(right). Chlase activity toward Chl *a* (O) and 280-nm absorbance (dashed curve) for the final chromatographic purification step on the Sephacryl S-200HR column.

Table 1 summarizes the result of successive purifications of Chlase from *C. regularis*. The amount of protein at each stage was determined by the method of Bradford.[12] In the final stage, the specific activity is 47.3 U/mg-protein, and represents a 13300-fold purity enhancement from the crude butanol extract. This is significantly superior to those in previous reports, *e.g.*, a 738-fold purification (activity 0.96 U/mg) from *C. protothecoides*[6] or 870-fold purification (0.48 U/mg) from greened rye seedlings.[13]

Table 1. Purification of Chlorophyllase from *Chlorella regularis*

Purification stage	Activity mU	Protein mg	Specific activity mU/mg	Purification factor (fold)	Yield %
Butanol extract	11500	3220	3.56	1	100
$(NH_4)_2SO_4$ precipitation	9630	2150	4.49	1.26	84
Toyopearl HW65-F	8160	125	65.1	18.3	71
Butyl-Toyopearl 650C	4580	3.62	1270	355	40
DEAE-Toyopearl 650S	2450	0.273	8980	2520	21
Sephacryl S-200HR	568	0.012	47300	13300	5

Figure 4 compares the Chlase activity to Chl *a* and *b*, Phe *a* and *b*, and their 13^2-epimers in 0.05% Triton X-100 aqueous solution, for the enzyme-containing solution obtained by the final Sephacryl S-200HR column

chromatography. As is clearly seen the enzyme is active exclusively to the non-primed (13^2R-form) pigments. The insignificant activity toward the epimers, being 2% or below of those for the non-primed pigments, is most probably due to their gradual epimerization[14] followed by enzymatic hydrolysis of the resulting 13^2R counterparts during incubation. Similar features were noted also in 30% acetone: *e. g.*, by 10-min incubation under the conditions of Fig. 4, Pheo *a* gave 2.2 nmol of Pheid *a*, while the amount hydrolyzed was well below 0.1 nmol for the four primed pigments. By the activity assay accompanying chromatographic purification of Chlase as illustrated in Figs. 2 and 3, use of Chl *a*' in place of Chl *a* did not reveal the presence of any activity peak.

These findings demonstrate that the Chlase from *C. regularis* neatly recognizes the $C13^2$ stereochemistry of chlorophyll pigments. The failure to detect any enzyme specifically hydrolyzing Chl *a*', which is present at the photosystem I core and is probably a key component for driving photosynthesis,[3] suggests that an epimerization step precedes the enzymatic degradation of this pigment *in vivo*.

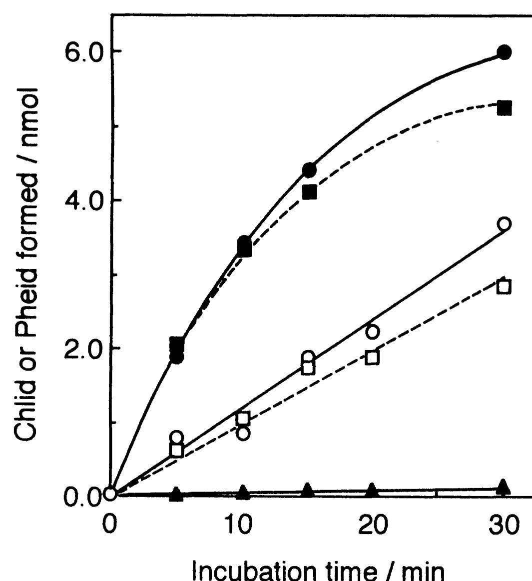

Fig. 4. Time courses of the enzymatic hydrolysis in 0.05% Triton X-100 aqueous solution. ●: Phe *a*, ■: Phe *b*, ○: Chl *a*, □: Chl *b*, ▲: Phe *a*', Phe *b*', Chl *a*', and Chl *b*'.

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