Plant & Cell Physiol. 22(4): 717–725 (1981)

Purification and Properties of Soluble Chlorophyllase from Tea Leaf Sprouts

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Soluble chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) was purified 650-fold from tea leaf sprouts by ammonium sulfate fractionation and gel filtration through Sephadex G-200 and Sepharose CL-6B. The purified enzyme showed two bands on polyacrylamide gel electrophoresis and the specific activity was 2.6 μmol chlorophyll *a* hydrolyzed min^{-1} mg^{-1} of protein. The molecular weights determined by Sepharose CL-6B were 910,000 and 350,000, indicating high molecular aggregates. The subunit molecular weight estimated by sodium lauryl sulfate-polyacrylamide gel electrophoresis was 38,000. The isoelectric point was 3.9. The optimum pH was 5.5 in acetate buffer and the K_m value for chlorophyll *a* was 10 μM. This enzyme did not require a thiol compound nor metal ion such as Mg^{2+}.

Key words: Chlorophyll — Purification (chlorophyllase) — Soluble chlorophyllase — Tea leaves — *Thea sinensis*.

Chlorophyllase, which has been known since 1913 (Willstätter and Stoll 1913), is widely distributed in higher plants (Ardao and Vennesland 1960, Shimizu and Tamaki 1963, Bacon and Holden 1970, McFeeters et al. 1971, Moll and Stegwee 1978) and algae (Barrett and Jeffery 1964, Böger 1965, Terpstra 1978) and catalyzes the hydrolysis of chlorophylls (Holden 1976) and transesterification of phytol and geranyl geraniol (Chiba et al. 1967, Wellburn 1970, Ellsworth 1971, Ichinose and Sasa 1973, Holden 1976). Chlorophyllase has been generally regarded as a membrane-bound enzyme, since it can be obtained in a soluble form only after treatment with detergent or organic solvent.

In the past, only two membrane-bound chlorophyllases have been purified to homogeneity, and only limited information is available about its physiological role. Previously, we reported the catalytic properties of *Chlorella* enzyme related to chlorophyll formation and the physical properties of the highly purified preparation (Chiba et al. 1967, Aiga and Sasa 1970, Ichinose and Sasa 1973, Tamai et al. 1979a, Shioi et al. 1980).

In contrast to the membrane-bound enzyme, Ogura (1969, 1972) first demonstrated, using young tea leaf sprouts, the presence of a soluble chlorophyllase, which was easily extractable in the supernatant after 100,000 $\times g$ centrifugation of the homogenate. He reported seasonal changes in the soluble and membrane-bound enzyme activities and comparison of catalytic properties between both partially purified preparations.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

This paper reports the catalytic and physical properties of highly purified soluble chlorophyllase from tea sprouts.

Materials and Methods

Chemicals—Sepharose CL-6B, Sephadex G-200 and Pharmalyte (pH 2.5–5.0) were purchased from Pharmacia Fine Chemicals (Sweden). All of the marker proteins used for the determination of molecular weight were obtained from Sigma Chemicals Co. (U.S.A.). All other reagents and solvents were of reagent grade.

Chlorophyll *a* was extracted from spinach leaves with 80% acetone and was further purified by the dioxane method (Iriyama et al. 1974) according to Perkins and Robert (1962) as described previously (Shioi et al. 1980).

Plant—Tea leaf sprouts (*Thea sinensis* L. Y-2) were harvested from April 24 to May 10 from a farm in Shintomi, Miyazaki Prefecture.

Assay of enzyme activity and protein concentration—The enzyme activity was determined by measuring the decrease of chlorophyll *a* concentration in the *n*-hexane layer, after partitioning of chlorophyll *a* and chlorophyllide *a* between *n*-hexane and aqueous acetone layers, according to the method of Chiba et al. (1967). The initial rate of the activity was estimated from the samples taken at 0, 3, 6, 9 and 12 min after the start of the reaction. In the partitioning procedure, the pH value of the acetone layer was adjusted to 7.5 because, in the lower pH range, some of the chlorophyllide *a* was transferred to the *n*-hexane layer as pointed out by McFeeters et al. (1971). The portions of chlorophyllide *a* in the *n*-hexane layer were about 16% at pH 5.5 and less than 3% at pH 7.5. For pH-activity studies, as the presence of acetone in the reaction mixture raises the pK of the buffer, we indicated the final pH values after addition of acetone. A standard reaction mixture contained in 2 ml, 40 mM acetate buffer (pH 5.0), 0.06 μmol of chlorophyll *a* dissolved in acetone (final acetone concentration was 25%), and the enzyme preparation (final pH of the mixture was 5.5). One unit of the enzyme was defined as the amount of the enzyme hydrolyzing 1.0 nmol of chlorophyll *a* per min. Specific activity was expressed as unit per mg protein. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

For estimation of the stoichiometry of the reaction, the amount of chlorophyllide *a* (product) in the acetone layer was determined after the product was transferred to ethyl ether. The millimolar extinction coefficient used for chlorophyllide *a* in ethyl ether was 91.1 at 660 nm which is the value for chlorophyll *a* (Comar and Zscheile 1942), because the spectral pattern of chlorophyllide *a* is essentially identical with that of chlorophyll *a* in the 340–700 nm range (Aiga and Sasa 1970).

Purification of tea sprout chlorophyllase—The purification procedures were essentially the same as the method of Shioi et al. (1980), except for *n*-butanol solubilization. All procedures were carried out at 0–4°C.

Extraction: Fresh tea sprouts (about 160 g) were homogenized in a Waring blender with about 270 ml of 40 mM phosphate buffer (pH 7.2) and the homogenate was filtered through six layers of gauze. The filtrate was centrifuged successively at 15,000 × *g* and 100,000 × *g* for 30 min each, and the supernatant (370 ml) was retained as the crude extract. The resulting solution was divided into small portions (74 ml each) and stored at −15°C in the dark until use.

Dialysis: Crude extract (74 ml) was dialyzed overnight against 10 mM phosphate buffer (pH 7.2) and centrifuged at 15,000 × *g* for 20 min. The supernatant was retained as the dialyzed extract for further purification. This process was necessary to obtain a reproducible and high recovery in the next ammonium sulfate fractionation step.

Ammonium sulfate fractionation (0–35% saturation): Ammonium sulfate was added to the supernatant to 35% saturation. After stirring for 1 hr, the precipitate, collected at 12,000 × *g* for 20 min, was dissolved in a small volume of 20 mM phosphase buffer (pH 7.2). The solution was clarified by centrifugation at 12,000 × *g* for 20 min.

Sephadex G-200 gel filtration: The ammonium sulfate fraction was applied to a column of Sephadex G-200 (2.2 × 55 cm) equilibrated with 20 mM phosphate buffer (pH 7.2), and eluted with the same buffer. The active fractions were pooled and concentrated with Minicon B 15 (Amicon Co., U.S.A.). This procedure removed most of the brown substances and low molecular weight proteins.

First Sepharose CL-6B gel filtration: The concentrated enzyme solution was applied to a column of Sepharose CL-6B (1.5 × 40 cm) equilibrated with 20 mM phosphate buffer (pH 7.2) containing 20 mM NaCl, and eluted with the same buffer. As shown in Fig. 1, chlorophyllase was separated from high molecular weight proteins as reported previously (Shioi et al. 1980). The active fractions were pooled and concentrated with Minicon B 15.

Second Sepharose CL-6B gel filtration: The concentrated enzyme solution was again applied to the same column of Sepharose CL-6B in a manner similar to that described above. The active fractions were pooled and concentrated with Minicon B 15. The purified enzyme retained 70–80% of its activity after storage for two months at −15°C in the dark.

Electrophoresis—Polyacrylamide gel electrophoresis of the purified enzyme was carried out using 8% gel in Tris-glycine buffer (pH 9.0) according to the procedure of Davis (1964).

Molecular weight—The molecular weight of the non-dissociating enzyme was determined by gel filtration through Sepharose CL-6B (1.5 × 65 cm) at 4°C. Bovine serum albumin (mol wt 67,000), γ-globulin (160,000), catalase (250,000) and ferritin (540,000) were used as standards. The moving phase consisted of 20 mM potassium phosphate buffer (pH 7.2) containing 100 mM NaCl. The void volume was estimated with Blue Dextran 2,000. Protein in each fraction was monitored by measuring the absorbance at 280 nm. Catalase and ferritin were estimated by its activity and by its absorbance at 430 nm, respectively. Chlorophyllase was measured by its enzyme activity. The subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (1969). The purified enzyme was treated with 2% SDS at 50°C for 4 hr then at 30°C for 16 hr. The following proteins were used as markers: cytochrome *c* (mol wt 12,400), chymotrypsinogen A (25,000), ovalbumin (43,000) and bovine serum albumin (67,000).

Isoelectric point—The isoelectric point was determined by the isoelectric focusing procedure using an Ampholine column and Pharmalyte (pH range 2.5–5.0) at 4°C for 44 hr, as described previously (Tamai et al. 1979b).

Results and Discussion

Enzyme purification

The purification process is summarized in Table 1. The enzyme was purified about 650-fold at the second Sepharose CL-6B gel filtration step, generally according to our purification procedure used for *Chlorella* cells (Shioi et al. 1980). The present recovery (9.6%) was lower than that in the case of *Chlorella* cells, perhaps because phenolic substances in tea sprouts hamper the purification.

Fig. 1 shows a typical elution pattern of the enzyme during the first Sepharose CL-6B gel filtration, exhibiting one main peak (fraction No. 20) and one minor shoulder (fractions around No. 16). Subjecting the concentrated active fractions (14–22) to the second Sepharose CL-6B gel filtration gave the same elution profile as that observed in the first gel filtration. The concentrated active fractions after the second gel filtration (14–22) were used as the purified enzyme for the later experiments, unless otherwise noted.

Polyacrylamide gel electrophoresis (8% acrylamide) of all enzyme preparations from the peak, shoulder and combined fractions showed two protein bands with the activity as shown by the assay of cut gels (1 mm thick) (Fig. 2). The two bands seem to be due to two types of the enzyme, presumably with different molecular weights.

Molecular weight and isoelectric point

The molecular weights of the purified enzyme were determined to be 910,000 (corresponding to the shoulder in Fig. 1) and 350,000 (the peak in Fig. 1) by Sepharose CL-6B gel filtration.

The pattern of SDS-gel electrophoresis of the purified enzyme was changed by the condition of enzyme treatment with SDS. Several protein bands due to different molecular species appeared after mild treatment (30°C for 2 hr) and a single band after drastic treatment (50°C for 4 hr and 30°C for 16 hr).

The subunit (minimum) molecular weight of the purified enzyme was deter-

Table 1 Purification of soluble chlorophyllase from tea leaves

Purification step	Total protein (mg)	Total activity (units[a])	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Crude extract[b]	558	2271	4.07	1	100
Dialysis	269	1629	6.06	1.5	71.7
Ammonium sulfate (0–35% saturation)	78.7	1379	17.5	4.3	60.7
Sephadex G-200	1.10	809	735	181	35.6
First Sepharose CL-6B	0.33	349	1058	260	15.4
Second Sepharose CL-6B	0.083	219	2639	648	9.6

[a] One unit of the enzyme activity was defined as the amount of the enzyme hydrolyzing 1.0 nmol of chlorophyll *a* per min under the conditions given in **Materials and Methods.**

[b] Purification was started from 74 ml of the crude extract corresponding to 32 g of the leaves in fresh weight.

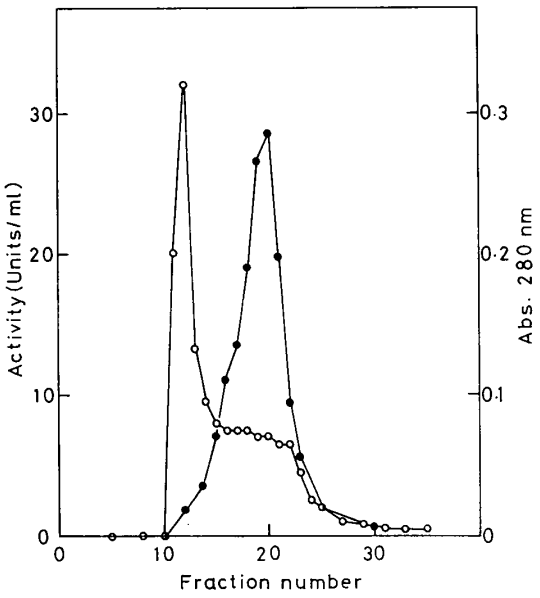

Fig. 1

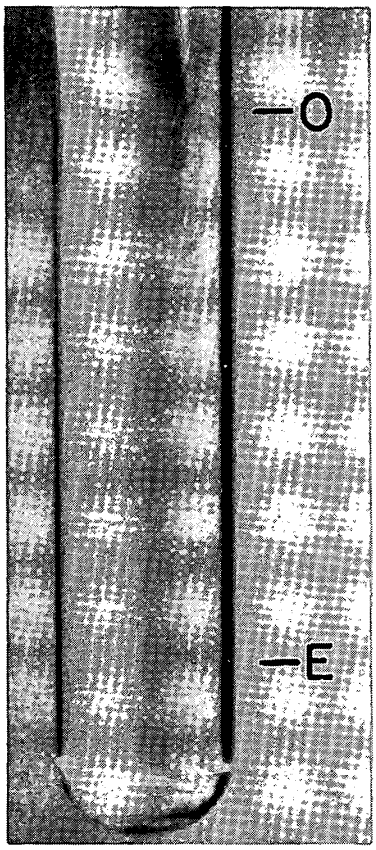

Fig. 2

Fig. 1 First Sepharose CL-6B gel filtration. The column (1.6×40 cm) was equilibrated with 20 mM phosphate buffer (pH 7.2) containing 20 mM NaCl and eluted with the same buffer at a flow rate of 10 ml per hr. Fractions (3 ml) were collected. ○, absorbance at 280 nm; ●, chlorophyllase activity.

Fig. 2 Polyacrylamide gel electrophoresis of the purified chlorophyllase. Electrophoresis was carried out in Tris-glycine buffer (pH 9.0) using 8% acrylamide gel. The protein (20 μg) was stained with Amino black 10B for 1 hr at 30°C. O, origin of the gel; E, end of the gel.

mined to be 38,000, which was the same as those from *Chlorella* (38,000), *Phaeodactylum* (38,000) and sugar beet (30,000–38,000) (Fig. 3).

All the above observations suggest aggregating characteristics of tea sprout chlorophyllase as already reported for chlorophyllases from sugar beet (Bacon and Holden 1970), wheat (Ellsworth 1971), *Phaseolus* (Moll and Stegwee 1978) and *Chlorella* (Tamai et al. 1979a). Therefore, a reliable estimation of the molecular weight of the enzyme would be difficult using usual procedures such as molecular sieving or polyacrylamide gel electrophoresis. The two enzymes separated by Sepharose CL-6B gel filtration were found to have the same pH optimum, K_m value, isoelectric point and subunit molecular weight, which may have resulted from non-specific aggregation. However, another possibility of the existence of two isozymes, each forming a high molecular aggregate, can not be denied.

As shown in Fig. 4, the isoelectric point was estimated to be 3.9 using Pharmalyte (pH range 2.5–5.0), while the pI for *Chlorella* enzyme was slightly different (pI 4.5) (Tamai et al. 1979a).

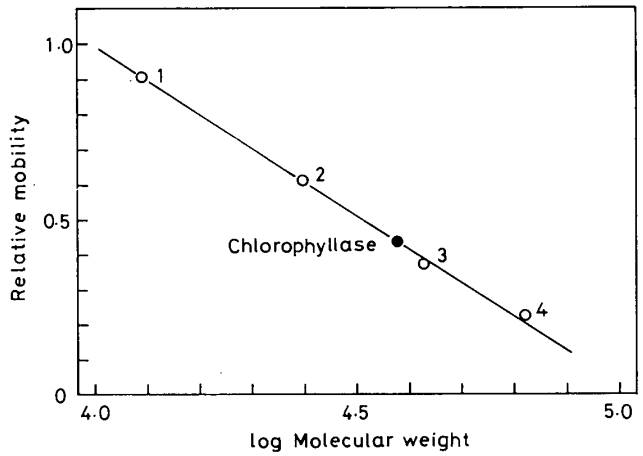

Fig. 3 Determination of the subunit molecular weight of the purified chlorophyllase. The protein (30 μg) was applied to the gel after dissociation in 2% SDS and 5% 2-mercaptoethanol. Molecular weight was calculated from mobility relative to known markers. 1, cytochrome *c*; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin.

Stoichiometry, pH optimum and K_m

The ratio of chlorophyll *a* (substrate) consumption and chlorophyllide *a* (product) formation was estimated to be practically 1 : 1 during the reaction. The result clearly shows that the reaction is catalyzed by chlorophyllase.

The optimum pH of tea sprout enzyme was 5.5 in acetate buffer (Fig. 5), which is similar to the pH 5.8 for the crude extracts prepared by Ogura (1978). The optimum pH of chlorophyllase has been reported for various degrees of purification from several plants and algae: Ailanthus, pH 4.5 (McFeeters et al. 1971); sugar beet, pH 7.1 (Bacon and Holden 1970); tobacco, pH 7.0–7.5 (Shimizu and Tamaki 1963); *Chlorella vulgaris*, pH 7.2–7.3 (Böger 1965); *Chlorella protothecoides*, pH 6.0–8.5 (Tamai et al. 1979a).

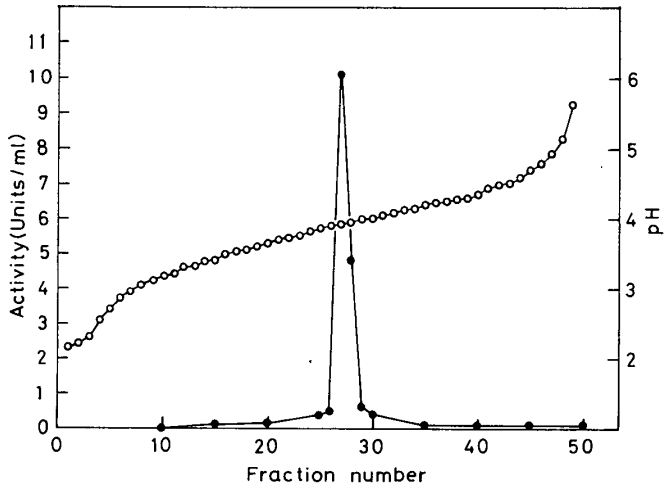

Fig. 4 Isoelectric focusing of the purified chlorophyllase. Electrophoresis was carried out for 44 hr at 4°C at 800–900 V in an Ampholine column (110 ml) using Pharmalyte in the pH range of 2.5–5.0. ●, chlorophyllase activity in 2-ml fractions; ○, pH measured at 4°C.

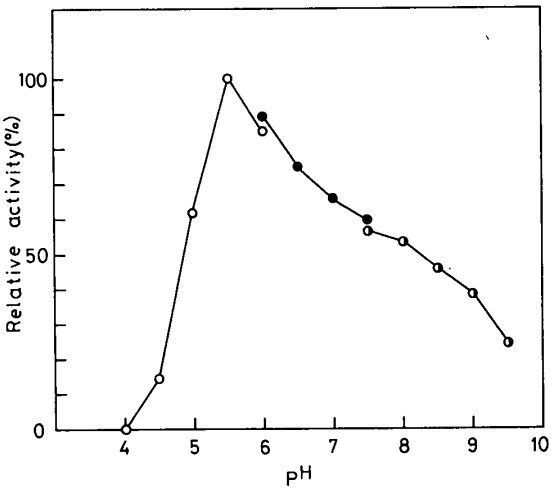

Fig. 5 pH Optimum of the purified chlorophyllase. The enzyme assay was done in 20 mM buffer at the indicated pH values. ○, acetate buffer; ●, potassium phosphate buffer; ◑, phosphate-borate buffer.

From the triplicate data at seven concentrations of chlorophyll *a* from 3 to 30 μM, the K_m value was 10 μM; this agrees fairly well with the 7.0 μM found by Ogura (1972).

Temperature dependency and thermal stability

Soluble chlorophyllase of tea sprouts showed maximum activity at 45°C under our conditions (Fig. 6). The Arrhenius plot of the activity was biphasic with a break occurring at 30°C. Activation energies were calculated as 18,000 cal mol^{-1} (10–30°C) and 3,500 cal mol^{-1} (30–45°C). The same situation has been reported for *Chlorella* enzyme (Shioi et al. 1980).

The thermal stability was examined by incubation of the enzyme prior to assay.

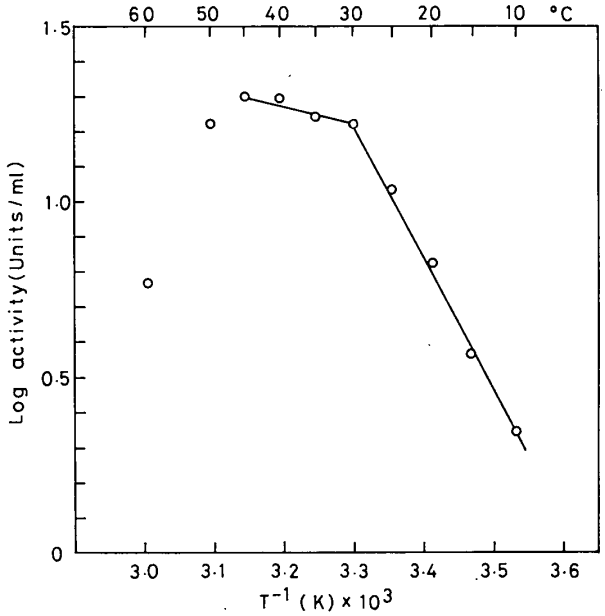

Fig. 6 Arrhenius plot of the activity of the purified chlorophyllase. The assay was carried out at the indicated temperatures. The reaction mixture and other assay conditions are described in **Materials and Methods**.

The results showed that the enzyme was reasonably stable at 50°C for 5-min incubation (6% inactivation) and about 35% of the activity was retained after 5-min incubation at 70°C.

Effect of SH reagents and salts

Terpstra (1978) has reported that dithiothreitol and $MgCl_2$ activate the chlorophyllase activity in *Phaeodactylum* membrane fractions. In the case of purified soluble tea chlorophyllase, no pronounced effect was observed by the addition of 1 mM dithiothreitol (11% inhibition). The enzyme activity was slightly inhibited by 1 mM *N*-ethylmaleimide (7% inhibition) but not affected by 0.5 mM PCMB. $MgCl_2$ (1–10 mM) had no effect on the purified enzyme (1–2% activation). Chelating agents, EDTA (1 mM) and *o*-phenanthroline (1 mM), also showed no effect (0–5% inhibition). These results indicate that tea sprout chlorophyllase does not require SH activators and metal ions such as Mg^{2+} to exhibit maximum activity.

Until now, soluble chlorophyllase has been detected only in tea leaves and *Phaeodactylum* (Terpstra 1978) among plant materials. We obtained a highly purified preparation of soluble chlorophyllase from tea sprouts, and determined the catalytic and physical properties. Catalytic properties of our purified enzyme were essentially similar to those of the enzyme partially purified by Ogura and Takamiya (1966) and Ogura (1969, 1972), except for susceptibility to PCMB, which inhibited Ogura's enzyme but had no effect on ours. Although in respect to extractability, soluble chlorophyllase from tea sprouts is quite different from other membrane-bound enzymes, no significant difference was recognized between our enzymes and membrane-bound ones.

On the participation of chlorophyllase in chlorophyll metabolism, no general agreement has yet been reached. The relationship between soluble and membrane-bound chlorophyllases must also be the subject of future research.

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

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(Received January 26, 1981; Accepted April 3, 1981)