0273-2289/95/5301—0011$07.40

Purification and Characterization of Chlorophyllase from Alga *Phaeodactylum tricornutum* by Preparative Native Electrophoresis

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Received April 26, 1994; Accepted August 2, 1994

ABSTRACT

The partially purified chlorophyllase, obtained from the alga *Phaeodactylum tricornutum*, was further purified by preparative native gel electrophoresis. The purification procedure provided the recovery of large amounts of a single purified chlorophyllase fraction. However, the electrophoretic analyses of the purified enzymatic fraction under denaturing conditions demonstrated the presence of two bands with mol wt of 43 ± 3 and 46 ± 3 kDa. The purification procedure resulted in 2- and 195-fold increases in chlorophyllase activity compared to that of the partially purified and crude enzymatic extracts, respectively. The optimum pH for chlorophyllase hydrolytic activity was found to be 8.0. The optimum incubation time and temperature for the hydrolytic activity of the purified chlorophyllase were found to be 2 h and 31°C, respectively. The optimum concentrations of magnesium chloride and dithiothreitol, used as activators, were 4 and 5 m*M*, respectively. The addition of individual plant membrane lipids, including phosphatidylcholine, phosphatidylglycerol, and β-carotene, to the reaction media increased the enzyme activity markedly. The purified enzyme fraction displayed preferential specificity toward

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selective substrates with an order of activity as follows: purified chlorophyll *b* > purified chlorophyll *a* > partially purified chlorophyll > crude chlorophyll. Diisopropyl fluorophosphate and phytol, respectively, showed noncompetitive and competitive inhibitory effects on chlorophyllase activity with K_i values of 0.78 m*M* and 3.75 μM, respectively.

Index Entries: Purification; preparative electrophoresis; chlorophyllase; *Phaeodactylum tricornutum*.

INTRODUCTION

Chlorophyllase (chlorophyll-chlorophyllido hydrolase, EC 3.1.1.14) is an intrinsic membrane glycoprotein located in the photosynthetic membrane of higher plants and algae (*1*). Chlorophyllase has been regarded as a membrane-bound enzyme, since it can be obtained in a soluble form only after treatment with detergents or organic solvents (*2*). Chlorophyllase was released as protein–chlorophyllide complex from *Phaeodactylum* photosynthetic membrane fragments using low-salt aqueous medium for incubation (*3*). Kermasha et al. (*4*) reported on the biomass production of the alga *Phaeodactylum tricornutum*, as a source for chlorophyllase. However, difficulties in the study of chlorophyllase were mainly the result of the insolubility of both enzyme and substrate in aqueous media (*5*).

Many studies have been directed to characterize the properties of chlorophyllase from higher plants and algae using crude, partially purified, and purified enzyme extracts (*4,6–8*). Chlorophyllase-catalyzed chlorophyll was greatly enhanced by the addition of magnesium and dithiothreitol (*1,4*). A number of membrane enzymes have been shown to be regulated by lipids (*9*). Lambers et al. (*10*) indicated that certain membrane lipids are required for chlorophyllase activity. The effect of inhibitors on chlorophyllase activity has been investigated (*4*). Levadoux et al. (*11*) showed that phytol has a strong competitive inhibitory effect on chlorophyllase activity.

Khalyfa et al. (*8*) reported the use of a preparative isoelectric focusing system for the purification of chlorophyllase, however, the presence of residual polyionic substances, the ampholines, associated with the purified enzymatic fraction limited the utility of such a technical process for the purification of chlorophyllase at the preparative scale.

Many attempts have been made to design continuous elution devices suitable for routine protein purification, in which bands emerging from bottoms of electrophoresis gels are swept away to fraction collectors (*12*). Bollag and Edelstein (*13*) indicated that polyacrylamide gel electrophoresis (PAGE) has been widely used in the fractionation of proteins and enzymes. Although electrophoresis is normally used for protein separation, on a small scale, it was also used on a large scale (*14,15*).

This work is a part of ongoing research work (*4,8,16*) aimed at the biomass production, purification, and characterization of chlorophyllase from alga *P. tricornutum*. As far as the authors are aware, the purification and characterization of chlorophyllase by preparative native electrophoresis have not been reported previously. The objective of this study was to optimize the use of Prep-Cell electrophoresis system (Model 491, Bio-Rad Laboratories, Richmond, CA), with continuous-elution process, for the purification of chlorophyllase from alga *P. tricornutum*, to characterize the purified enzymatic fraction in terms of its optimum pH, electrophoretic profile, molecular weight, kinetic parameters including K_m and V_{max} values, and to study the effects of incubation temperature and time as well as the addition of L-α-phosphatidylcholine, L-α-phosphatidyl-DL-glycerol, β-carotene, magnesium chloride, dithiothreitol, phytol, and diisopropyl fluorophosphate on the hydrolytic activity of the purified enzymatic fraction.

MATERIALS AND METHODS

Materials

Culture Growth and Harvesting Conditions

The biomass production of alga *P. tricornutum* Bohlin (*Bacillariphyceae*), used throughout this study, was carried out according to Kermasha et al. (*4*).

Methods

Extraction and Partial Purification of Chlorophyllase

Chlorophyllase was extracted from *P. tricornutum*, at the stationary phase, and partially purified according to the procedure described previously by Kermasha et al. (*4*).

Protein Determination

The purified chlorophyllase fraction was assayed for protein content, using a modification of the Lowry method (*17*). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard to establish a calibration curve.

Preparative Electrophoresis

The partially purified enzyme was subjected to further purification by preparative Prep-Cell electrophoresis system (Model 491; Bio-Rad Laboratories), using cylindrical gel tube (3.7 × 13.5 cm). The preparative native PAGE was performed according to a modification of the method described by Maurer (*18*). All procedures were carried out at 4°C, unless otherwise specified. Two types of gels (separation and stacking) were prepared and

used throughout this electrophoresis process. The total percentage concentration (T%) of both monomers, acrylamide, and bis(*N,N'*-methylene-bisacrylamide) for the separation gel was 30.8% and 15% for the stacking gel. The percentage concentration of the crosslinker relative to the total concentration (C%) was 2.6% for the separation gel and 20% for the stacking gel.

The separation gel (100 mL) was degassed for 15 min, cast in the cylindrical tube (11 cm), and allowed to polymerize overnight at room temperature. The temperature of polymerization was maintained by continuous circulation of water using recirculation pump at flow rate 80–100 mL/min through a ceramic cooling finger located at the center of the gel. The stacking gel (24 mL) was degassed for 10 min, introduced and cast at the top of cylindrical gel tube (2.5 cm), and polymerized for 2 h. After polymerization, the cylindrical gel tube was removed from the casting stand very carefully to obtain a smooth lower surface; trapped air bubbles may cause a pitted gel surface that will result in uneven elution of proteins from the gel. The cylindrical gel tube containing separation and stacking gels was fixed on the elution chamber base equipped with the support frit, the dialysis membrane, and the elution frit. The frits and dialysis membrane were soaked over night in Tris-glycine (5 m*M*, pH 8.0) buffer prior to use.

The partially purified chlorophyllase (18 mg protein) suspension was prepared in 1 mL stacking gel and homogenized in a tissue grinder (Wheaton, Millville, NJ). The homogenized enzyme was then applied on the top of the stacking gel and polymerized for 30 min at 4°C.

The Tris-glycine elution buffer (5 m*M*, pH 8.3) was degassed using filter (0.45 μm, Millipore, Canada, Ltée) and pulled from the elution buffer reservoir to the elution buffer port on the chamber cap. The flow of the elution buffer was directed through a series of channels in the elution chamber gasket to the perimeter of the elution frit.

The initial conditions of electrophoresis were 325 V, 18 W, and limited to 45 mA; however, at the end of the run, these values were 480 V, 26 W, and 45 mA. In order to lower the heat generated during the electrophoresis time (7 h), a recirculation pump provided a flow rate of 80 mL/min of buffer Tris-glycine solution (5 m*M*, pH 8.3) at 4°C through a ceramic cooling finger. As the protein fractions migrated off the gel, they were washed to the center of the frit and through the elution tube in the center of the cooling core. The protein fraction (2 mL), eluted at a flow rate of 1 mL/min at 280 nm, was monitored by its absorbance at 280 nm using a UV detector (Model, 2138, LKB, Co.). The separated fractions were collected, and desalted according to the method described by Khalyfa et al. (*8*), using gel-filtration Econo-pac 10DG (Bio-Rad, Laboratories). The desalted purified enzymatic fraction was lyophilized, and subjected to further characterization and kinetic studies.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemmli (*19*), using minigels (5 × 4 cm) prepared with concentration of total monomers (T) = 12.5%

and concentration of crosslinker (C) = 3%. The protein standards and the purified fraction were incubated in Laemmli reducing buffer (*19*) at 90°C for 10 min. Electrophoretic separation was performed for 30 min at a constant current of 10 mA, using the PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden). The proteins were stained with silver nitrate according to the method described by Pharmacia (*20*). The standard curve was established with a mixture of mol-wt markers (Pharmacia), including lysozyme (M_r 14,400), trypsin inhibitor (M_r 21,500), carbonic anhydrase (M_r 31,000), ovalbumin (M_r 45,000), bovine serum albumin (M_r 66,200), and phosphorylase b (M_r 97,400).

Substrate Preparation

The partially purified chlorophyll, prepared from spinach leaves according to the procedure described previously by Khalyfa et al. (*16*) was used as substrate for the determination of chlorophyllase activity. The substrate selectivity of the purified chlorophyllase fraction was determined by using crude extract, partially purified chlorophyll (*16*), and commercial purified chlorophylls *a* and *b* (Sigma Chemical Co., St. Louis, MO). The stock solutions of substrates (1 mg/mL acetone) were prepared according to the method of Kermasha et al. (*4*). The solutions of substrates and subsequent dilutions were freshly prepared prior to the enzyme assay.

Enzyme Assay

The enzymatic assay was performed according to the method described previously (*4*). The reaction mixture was prepared in a 50-mL Erlenmeyer flask fitted with a ground-glass stopper. The mixture contained 1 mL enzyme suspension (0.45 μg protein) and 70 μL chlorophyll solution (14 μg chlorophyll). The initial amount of chlorophyll used as substrate was measured at 662 nm, whereas the absorbance of the lower layer containing the chlorophyllide fraction was measured at 667 nm. A control assay was performed, using all components except the enzyme fraction. One unit of chlorophyllase activity fraction is defined as the amount of enzyme that catalyzes the production of 1 μmol chlorophyllide/min. The specific activity of chlorophyllase fraction is defined as μmol of chlorophyllide formed/mg protein/min.

Effect of Acetone Concentration on Chlorophyllase Activity

The effect of acetone (BDH, Inc., Dermstadt, West Germany), ranging from 0 to 50%, on the hydrolytic activity of purified chlorophyllase was studied.

Effect of Activators on Chlorophyllase Activity

The effects of magnesium chloride and dithiothreitol (BDH, Inc., Dermstadt, West Germany), at various concentrations ranging from 0 to

10 m*M*, on the hydrolytic activity of purified chlorophyllase were also investigated.

Effect of Lipids on Chlorophyllase Activity

The effects of membrane lipids, L-α-phosphatidylcholine (PC), L-α-phosphatidyl-DL-glycerol (PG), and β-carotene (Sigma Chemical Co., St. Louis, MO), at various concentrations from 0 to 100 μg, on the hydrolytic activity of purified chlorophyllase were investigated. The lipid solutions were prepared in Tris-HCl buffer solution (pH 8.5, 0.02*M*) containing 20% acetone and stored in screw-cap brown vials under nitrogen to minimize oxidation.

Effect of Inhibitors on Chlorophyllase Activity

The effects of phytol (Sigma Chemical Co., St. Louis, MO) and diisopropyl fluorophosphate (Aldrich Chemical Co., Inc., Milwaukee, WI), at various concentrations ranging from 0 to 10 m*M*, on the hydrolytic activity of purified chlorophyllase were also investigated.

RESULTS AND DISCUSSION

Enzyme Purification

The purification of the partially purified chlorophyllase FII (Table 1) by preparative native polyacrylamide gel electrophoresis (PAGE) (Fig. 1), using Prep-Cell System (PCS), resulted in a single major enzymatic fraction. The results (Table 1) indicate that the recoveries of purified enzyme fractions FIII and FIV obtained, respectively, without and with the use of the Econo-pac 10DG column, were 73 and 21% of that of the crude enzyme extract FI. These results (Table 2) demonstrate that the use of preparative electrophoresis increased the chlorophyllase activity by 2- and 195-fold compared to that of the partially purified and crude enzymatic extracts, respectively. Although the increase in the chlorophyllase activity appeared to be limited, the technique used allowed the purification of relatively high amounts of proteins (18 mg) per run and the removal of cell debris.

Characterization of End Product

In order to monitor the removal of residual contaminants associated with the partially purified enzyme FII, a spectrophotometric scanning (350–700 nm) was carried out. The results (Fig. 2A) demonstrate the presence in enzymatic fraction (FII) of such contaminants, including carotenoids and chlorophylls with a maximum absorbance at 422 and 666 nm, respectively. However, Fig. 2B shows the absence of such contaminants

Table 1
Purification Scheme of Purified Chlorophyllase Fraction
Obtained from Alga *P. tricornutum* by Prep-Cell Electrophoresis

Fractions	Total protein,[a] mg	Total activity[b]	Specific activity, X10^{-4}	Recovery, %	Purification fold
Crude extract (FI)[d]	2246.1	4155.3	1.9	100.0	0.0
Partially purified (FII)[d]	94.2	15539.7	165.0	374.0	89.2
Preparative electrophoresis (FIII)[e]	16.4	3029.9	184.3	72.9	99.6
Preparative electrophoresis (FIV)[f]	2.4	878.5	360.0	21.1	194.6

[a] Protein concentration was determined according to a modification of Lowry method (17), using bovine serum albumin as standard.
[b] A unit of activity is defined by the formation of 1 μmol of chlorophyllide/min.
[c] Specific activity is defined as μmol product/mg protein/min.
[d] Crude extract and partially purified enzyme, as acetone extract, were prepared as described by Kermasha et al. (4).
[e] Fraction obtained by preparative electrophoresis without filtration on the Econo-pac™ 10DG column (Bio-Rad).
[f] Fraction obtained by preparative electrophoresis and filtered on a gel-filtration column (Econo-pac™ 10DG).

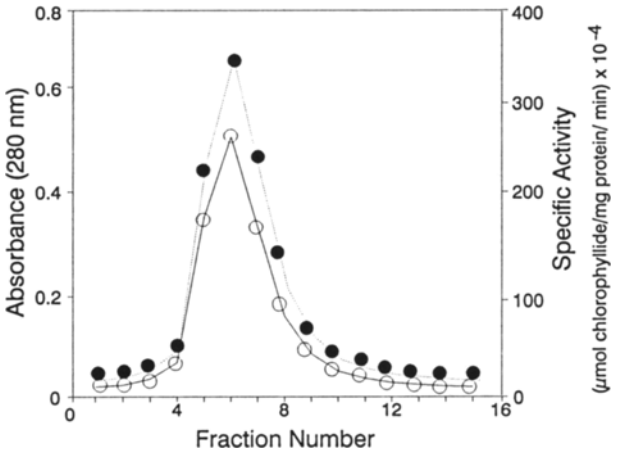

Fig. 1. Profile of preparative native PAGE separation of chlorophyllase of *P. tricornutum*, obtained by Prep-Cell system: (○) protein content and (●) chlorophyllase activity.

Table 2
Characterization of Purified Chlorophyllase Fraction
Obtained from Alga *P. tricornutum* by Prep-Cell Electrophoresis

Inhibitor	K_i	K_m[a]	$K_{m'}$[a]	V_{max}[b]	$V_{maxapp.}$[b]
Control[c]	—	2.1	—	800	—
DIFP[d]	0.78 m*M*	2.1	—	800	675
Phytol	3.75 μM	—	3.2	800	—

[a] K_m values are expressed as substrate concentration [S] $M \times 10^{-9}$.
[b] V_{max} values are expressed as μmol product/mg protein/min $\times 10^{-4}$.
[c] Enzymatic reaction without inhibitor addition, using chlorophyll as substrate.
[d] Diisopropyl fluorophosphate (DIFP).

in the purified fraction FIV. Using thin-layer chromatography analyses, Terpstra and Lambers (*21*) showed the association of residual contaminants of carotenoids, sulfolipids, and galactolipids with the partially purified chlorophyllase from *P. tricornutum*. In addition, Lambers et al. (*10*) reported that membrane proteins have been shown to contain some lipids after their purification.

The spectrophotometric scanning was also used to characterize the end products of the hydrolytic activity of purified chlorophyllase fraction (FIV). The results (Fig. 3A) demonstrate a maximum absorbance of 662 nm for the chlorophyll substrate, whereas (Fig. 3B) shows a maximum absorbance of 667 nm, characteristic of the end product chlorophyllide. The maximum absorbance of the end product chlorophyllide is similar to that reported by Khamessan et al. (*22*). However, these findings (Fig. 3B) are slightly different from those reported by Kermasha et al. (*4*), where

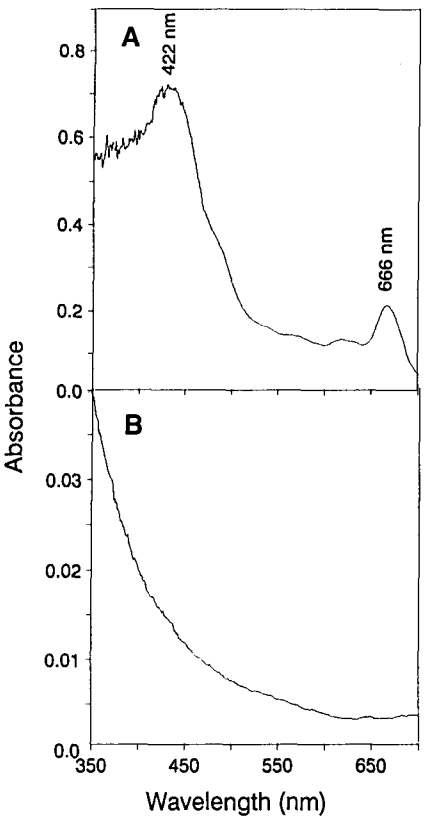

Fig. 2. Absorption spectrum of: (**A**) partially purified chlorophyllase FII (**B**) Prep-Cell purified enzyme fraction (FIV).

the maximum absorbance was found to be 670 nm. These slight differences may indicate a shift in maximum absorbance that could be the result of the effect of acetone.

Analytical Electrophoresis

The electrophoretic analyses of purified chlorophyllase fraction FIV under denaturing conditions demonstrate (Fig. 4A) the presence of two bands. The relative molecular weights of the two separated bands, estimated from log molecular-weight protein standards vs R_f, were determined to be 43 $\pm$ 3 and 46 $\pm$ 3 kDa. Lambers et al. (*3*) concluded that both active and inactive forms of chlorophyllase of *P. tricornutum* contain three glycopolypeptides, 130, 42, and 30 kDa, and two polypeptides, 18 and 14 kDa. Tarasenko et al. (*23*) indicated that although chlorophyllase protein complexes in chloroplast membranes contain a large number of polypeptides, there is only one common polypeptide at 67 kDa showing the enzymatic activity. A mol wt of 38 kDa was also reported for chlorophyllase obtained from *P. tricornutum* (*24*), sugar-beet leaves (*6*), and *C. protothecoides* (*25*).

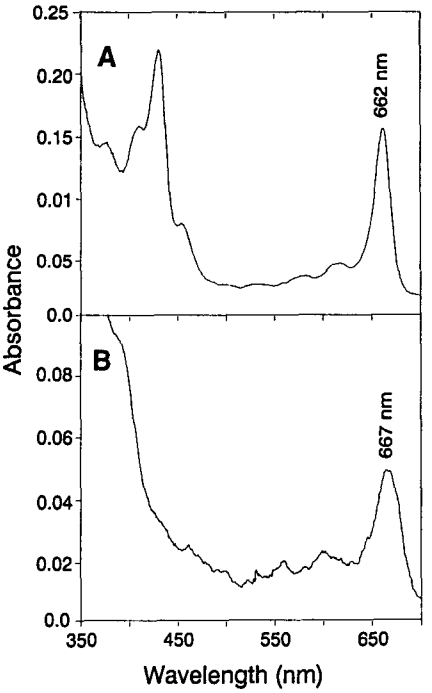

Fig. 3. Absorption spectrum of chlorophyllase enzymatic assay, using Prep-Cell system electrophoresis: (**A**) measurements of the substrate, chlorophyll, and (**B**) measurements of the end product, chlorophyllide.

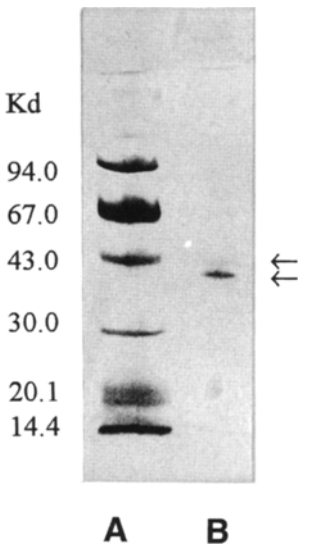

Fig. 4. SDS-PAGE electropherograms, using PhastSystem: (**A**) chlorophyllase-purified fraction obtained by Prep-Cell system electrophoresis and (**B**) low-molecular-weight protein standards.

Table 3
Optimum Parameters of Purified Chlorophyllase Fraction Obtained from Alga *P. tricornutum* by Prep-Cell Electrophoresis

Parameters	Optimum condition	Specific activity,[a] $\times 10^{-4}$
pH	8.0	70
Acetone	20%	83
Temperature	31°C	119
Time of incubation	2 h	140
Magnesium chloride	4.0 m*M*	152
Dithiothreitol	5.0 m*M*	172
Magnesium chloride and dithiothreitol	4.0 + 5.0 m*M*	184
Diisopropyl fluorophosphate	5 m*M*	6
Phytol	50 μM	42
Substrate specificity		
Crude chlorophyll[b]		9
Partially purified chlorophyll[b]		360
Purified chlorophyll *a*[c]		134
Purified chlorophyll *b*[c]		410

[a]Specific activity is defined as μmol chlorophyllide/mg protein/min.
[b]Crude and partially chlorophyll extracts were prepared as described previously by Khalyfa et al. (*16*).
[c]Commercial purified chlorophylls *a* and *b* were obtained from Sigma Chemical Co.

Tanaka et al. (*26*) reported a mol wt of 39 kDa for the chlorophyllase of green rye seedlings. A high molecular weight (150 kDa) was reported by Fernandez-Lopez et al. (*27*) for the chlorophyllase of citrus lemon leaves. These conflicting results could be the result of differences in sources of the enzyme or methods of purification and electrophoretic analyses.

Optimum pH

The results (Table 3) show that the optimum pH for the hydrolytic activity of the purified chlorophyllase fraction was found to be 8.0. This pH value is similar to that indicated previously by Khamessan et al. (*22*) for the partially purified chlorophyllase and close to pH 8.5 reported for the purified fraction FII′ (*8*). The optimum pH for the chlorophyllase activity in higher plants and algae was reported to range from pH 6.0 to 8.0 (*7*).

Effect of Acetone Concentration

The effect of acetone concentration on chlorophyllase activity shows (Table 3) that the presence of 20% (v/v) acetone in the reaction media increased the enzymatic activity by 18.5%. Garcia and Galindo (*28*) reported

that the hydrolytic activity of chlorophyllase was modified by acetone concentration. Chlorophyllase appeared to be the first enzyme reported to retain its enzymatic activity in organic solvents (*29,30*). Because of the poor substrate solubility of chlorophyllase-catalyzed reaction in aqueous media, Bacon and Holden (*6*) suggested that it required the presence of an organic solvent. The insolubility of chlorophylls in aqueous media could be expected to cause difficulty in measuring some of the physicochemical properties of the enzyme (*5,22*). The poor solubility of chlorophyllase and its substrate, chlorophyll, in aqueous solution can be overcome by using organic solvents, such as acetone and methanol, in the reaction media (*6*). The highest hydrolytic activities were demonstrated at 17.5, 25–32, 30, 35, 40, and 40–60% of acetone for chlorophyllase, obtained from *P. tricornutum* (*22*), citrus leaves (*28*), wheat seedlings (*31*), citrus lemon leaves (*27*), *Chlorella vulgaris* (*32*) and *Ailanthus altissima* (*7*), respectively. Fernandez-Lopez et al. (*27*) indicated that the chlorophyllase activity decreased when the acetone concentration was lower than 35%, and this was probably owing to the insolubility of chlorophyll. However, higher concentrations of organic solvents caused an enzymatic inhibition that could be attributed to protein precipitation (*22*). McFeeters et al. (*7*) reported that the activity of chlorophyllase from *A. altissima* was decreased in presence of 70% acetone.

Effect of Temperature and Time of Hydrolysis

The results (Table 3) show that the optimum temperature for the hydrolytic activity of the purified fraction is 31°C. The literature indicated that the optimum temperature for chlorophyllase activity ranged from 25 to 37°C (*4,27,31,33*). However, 82% of chlorophyllase activity was lost (data not shown) when the temperature rose to 35°C. Lambers et al. (*10*) indicated that a greater part of chlorophyllase activity was lost when the incubation time reached 37°C at 30 min.

The results (Table 3) demonstrate that the optimum incubation time for the bioconversion of chlorophyll into chlorophyllide is 2 h. These results are similar to those reported by Khamessan et al. (*22*) and different from those (9 h) indicated by Kermasha et al. (*4*). The difference in the optimum incubation time could be the result of the presence of 20% acetone.

Effect of Activators

The results (Table 3) show that the optimum concentrations of $MgCl_2$ and dithiothreitol, used as activators for the chlorophyllase activity, were 4 and 5 m*M*, respectively. However, these results are different from those reported for the optimum concentrations of $MgCl_2$, 2.0 and 10.0–12.0 m*M*, respectively, by Kermasha et al. (*4*) and Terpstra and Lambers (*21*). The optimum concentration of dithiothreitol (Table 3) is also different from those 3.0 and 6.7 m*M* reported by Kermasha et al. (*4*) and Terpstra (*34*),

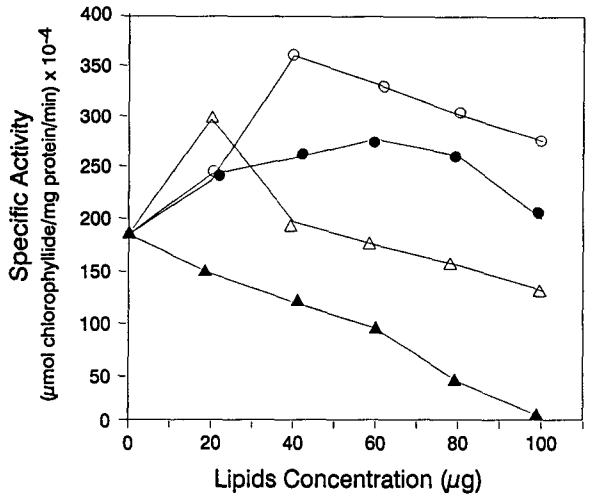

Fig. 5. The effect of lipid concentrations of chlorophyllase activity: L-α-phosphatidylcholine (△), β-carotene (●), L-α-phosphatidyl-DL-glycerol (○), and a mixture of L-α-phosphatidylcholine/β-carotene/L-α-phosphatidyl-DL-glycerol (20:40:60, w/w/w) (▲).

respectively. Lambers et al. (*10*) indicated that the metal ion is believed to induce, primarily, the conformational changes in the protein and, secondarily, to interact with the head groups of lipids that bind tightly to the protein for the formation of an active enzyme complex. Terpstra (35) suggested that the activating effect of combined $MgCl_2$ and dithiothreitol may indicate that chlorophyllase contains free sulfhydryl groups that are essential for the enzyme activity; this author suggested, hence, that the binding of sulfhydryl groups could provide the right enzyme conformation for the chlorophyllase catalytic activity.

Effect of Lipids

The results (Fig. 5) indicate that the addition of individual 20 μg PC, 40 μg β-carotene and 60 μg PC increased the specific activity of chlorophyllase from 184.52 × $10^{-4}M$ to 296.91 × $10^{-4}M$, 360.02 × $10^{-4}M$, and 277.08 × $10^{-4}M$, respectively. However, Terpstra and Lambers (*21*) suggested that chlorophyllase activity was increased by 50 and 35% with the presence of PG and β-carotene, respectively. Sandermann (*9*) indicated that the presence of certain lipids could play a role either as stabilizers or activators of the membrane enzymes. Terpstra and Lambers (*21*) suggested that lipids could affect the conformation of various membrane enzymes, such as chlorophyllase.

In addition, the results (Fig. 5) demonstrate that the addition of a mixture of optimum individual concentration (20:40:60, w/w/w) of PC, β-carotene, and PG to the enzymatic assay decreased the chlorophyllase activity. Lambers and Terpstra (*36*) reported that the negatively charged

lipid PG, either alone or mixed with other plant lipids, could also inactivate the chlorophyllase. Lambers and Terpstra (*36*) reported that the inhibition of chlorophyllase by PG can be abolished by the action of phospholipase A_2; these authors suggested that not only the PG head group, but also the fatty acid chain could play a role in the binding and inactivation of the enzyme.

Effect of Inhibitors

The results (Table 3) show that the activity of the purified chlorophyllase fraction was almost completely inhibited at 5 m*M* diisopropyl fluorophosphate (DIFP), whereas 50 μM of phytol decreased the enzymatic activity from 184×10^{-4} to 142×10^{-4}. The inhibitory effect of DIFP is somewhat similar to that reported for the partially purified enzyme (*4*), as well as to that indicated for the purified enzymatic fraction obtained by isoelectric focusing (*8*).

The K_i value, calculated from plotting $1/v$ vs inhibitor concentration [I], is a measure of the affinity of the inhibitor for the enzyme (*4*). The results (Table 2) show that the K_i value for DIFP was determined to be 0.78 m*M*; this value is lower than that (2.14 m*M*) reported for the purified enzyme fraction FII′ obtained by isoelectric focusing (*8*) and that (5.57 m*M*) for the partially purified chlorophyllase (*4*). The K_i value of phytol (Table 3), a product resulting from the conversion of chlorophyll into chlorophyllide, was determined to be 3.75 μM. Terpstra (*37*) suggested that phytol could exhibit an inhibitory effect on the chlorophyllase activity.

The Lineweaver-Burk plots (*38*) were also used to determine the effect of substrate concentration on the initial velocities of the hydrolytic activity of purified chlorophyllase. The K_m value (Fig. 6; Table 3) for the purified chlorophyllase in the presence and absence of DIFP was determined to be $2.1 \times 10^{-9} M$, a value close to the $2.3 \times 10^{-9} M$ reported for the purified chlorophyllase fraction obtained by preparative isoelectric focusing (*8*). The plots of $1/v$ vs 1/[S] (Fig. 6) demonstrate that DIFP is a noncompetitive inhibitor for the chlorophyllase activity of the purified enzyme; these results agree with those reported by Kermasha et al. (*4*) and Khalyfa et al. (*8*) for the partially purified and FII′ purified enzyme extract, respectively. Although irreversible inhibition was once categorized and tested as noncompetitive inhibition, it is now recognized as a distinct type of inhibition (*39*). The plots of $1/v$ vs 1/[S] (Fig. 6) indicate that phytol is a reversible inhibitor under the classification of competitive type owing to the change of K_m value and the unchanged V_{max} value. Therefore, a plot of V_{max} vs the total amount of enzyme $[E]_{Total}$ (Fig. 7) demonstrates that the presence of 5 m*M* DIFP should indicate clearly whether DIFP is an irreversible or a noncompetitive inhibitor.

The K_m' value calculated also (Fig. 6; Table 3) from Lineweaver-Burk plots of $1/v$ vs 1/[S], in the presence of phytol, was found to be $3.2 \times 10^{-9} M$. Levadoux et al. (*11*) reported that phytol has a strong competitive inhibi-

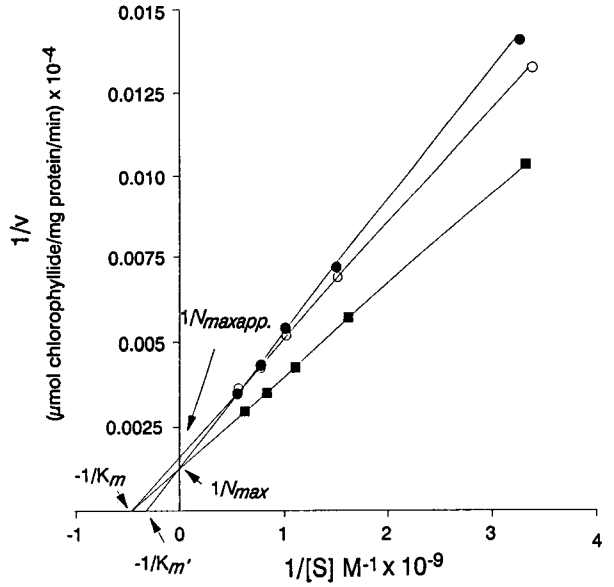

Fig. 6. Lineweaver-Burk plots ($1/v$ vs 1/[S]) of chlorophyllase activity of purified fraction obtained by Prep-Cell system electrophoresis by the addition of: diisopropyl fluorophosphate (●), phytol (○), and no inhibitor (■).

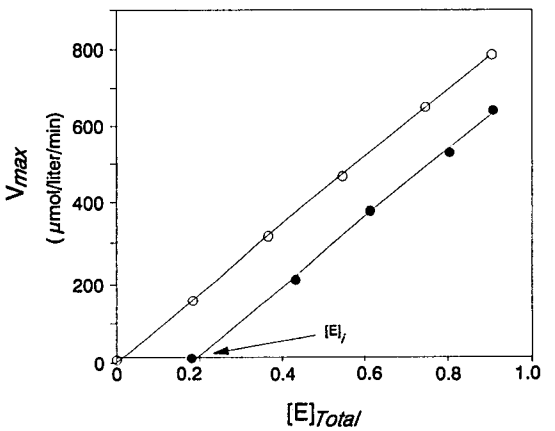

Fig. 7. Plots of V_{max} vs $[E]_{Total}$ of chlorophyllase activity of purified enzyme fraction obtained by Prep-Cell system electrophoresis: no inhibitor (○) and diisopropyl fluorophosphate (●).

tory effect on chlorophyllase activity; these authors also indicated that phytol was likely responsible for the limited hydrolysis of the high level of chlorophyll. The V_{max} was determined to be 800 × 10^{-4} (Fig. 7; Table 2), whereas the $V_{maxapp.}$ was 675 × 10^{-4} (Fig. 7B, Table 2); however, the $V_{maxapp.}$ value is higher than that reported (200 × 10^{-4}) for the purified chlorophyllase fraction FII′ (*8*).

Substrate Specificity

The substrate specificity of the purified chlorophyllase fraction was assayed for crude chlorophyll, partially purified chlorophyll, and commercial purified chlorophylls *a* and *b*. The results (Table 3) indicate that the purified enzyme fraction showed higher chlorophyllase activity toward the commercial purified chlorophyll *b* (410.30×10^{-4}) compared to those obtained with commercial purified chlorophyll *a* (133.90×10^{-4}), partially purified chlorophyll (360.04×10^{-4}) and with the crude chlorophyll (9.10×10^{-4}). However, Khalyfa et al. (*8*) reported that the chlorophyllase fractions FII′ showed limited activity for both commercial purified chlorophylls *a* and *b*, and that may be explained by the fact that the absence of chloroplast lipids could limit the enzyme activity (*21*).

CONCLUSION

The results gathered in this study showed that the use of preparative native PAGE Prep-Cell system (Model 491, Bio-Rad) is a convenient method for the purification of relatively large amounts of chlorophyllase from *P. tricornutum*, and for the removal of cell debris and residual chlorophylls and carotenoids, generally associated with the partially purified enzymatic fraction.

ACKNOWLEDGMENT

This research was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada.

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