IDENTIFICATION OF CHLOROPHYLLASE AS A GLYCOPROTEIN

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1. Introduction

Chlorophyllase (chlorophyll–chlorophyllidohydrolase EC 3.1.1.14), or its precursor, occurs in photosynthetic plant membranes. Relatively large amounts are found in the diatom *Phaeodactylum tricornutum* [1,2]. The physiological function of the enzyme, which is active only after cell disintegration, is still not clear. For the application of various physical techniques to the investigation of chlorophyllase, several milligrams of the enzyme were required. A suitable procedure for enzyme isolation was developed on the basis of earlier results [3]. In the course of these experiments it became clear that active chlorophyllase is a concanavalin A-reactive glycoprotein. The enzyme contains at least 3 different glycopeptides. Evidence is presented that one of these glycopeptides plays a role in the enzymatic activity.

2. Materials and methods

2.1. *Materials*

Reagents for preparing gels, including SDS, were purchased from Bio-Rad. Sephadex G-100 and concanavalin A–Sepharose 4B were supplied by Pharmacia. Fuchsin–sulfite solution was obtained from Sigma. All other chemicals used were of analytical quality.

2.2. *Cultivation of* Phaeodactylum tricornutum

Cells were cultured for ~12 days in artificial seawater, containing ~50 mg sodium silicate/litre, at 17°C. The flasks were illuminated with Philips 33 fluorescent tubes; the light intensity was approximately 7 mW/cm^2. The culture medium was aerated with air enriched with 5% CO_2.

2.3. *Isolation of chlorophyllase*

All procedures were done at 0–5°C unless otherwise stated.

(a) *Membrane preparation:* About 40–50 g algae (wet wt) were washed once with 0.02 M Tris–HCl buffer (pH 8.0) containing 2% NaCl, then suspended in 0.02 M Tris–HCl buffer (pH 8.0) containing 5×10^{-4} M EDTA (Tris–EDTA). The cells were disintegrated by passing them through a French press (620 kg/cm^2). After centrifugation at 10 000 × *g* for 15 min the brown supernatant, containing the photosynthetic membrane fragments, was centrifuged at 226 000 × *g* for 1 h. The precipitated membranes were washed 3 times with 0.01 M $Na_4P_2O_7$ (pH 7.4) [3–5], then once with 'Tris–EDTA' and resuspended in 'Tris–EDTA'.

(b) *Acetone precipitation of proteins:* One volume of membrane suspension was mixed with 4 vol. cold (–20°C) acetone. After 15 min in the refrigerator the precipitate was spun down, washed once with 80% acetone–20% 'Tris–EDTA', then extracted twice with ~50 ml 'Tris–EDTA'. The combined extracts were concentrated, under nitrogen pressure, on an Amicon YM-10 filter (cut-off at M_r 10 000) to ~6 ml final vol. Contrary to what was found with Amicon PM and UM filters, no significant adsorption of chlorophyllase to YM filters occurred.

(c) *Preparative polyacrylamide gel electrophoresis:*

The sample was subjected to electrophoresis on discontinuous gels containing 0.1% Triton X-100, according to [6]. The 'running gels' (12 each of 7.5 ml in tubes with an inner diameter of 13 mm), contained 5% acrylamide and 0.13% bisacrylamide, the 'stacking gels' (each 0.8 ml) contained 2.5% acrylamide and 0.625% bisacrylamide. Densities of the stacking gels

and of the sample were adjusted with glycerol. Electrophoresis was carried out at 10°C, the current being ~7 mA/tube. Carotenoids, often abundantly present in the sample, remained on top of the stacking gel. After electrophoresis those parts of the gels containing proteins with electrophoretic mobilities between 0.1 and 0.4 (in relation to a mobility of 1.0 of the tracking dye bromophenol blue) were cut out. Control experiments indicated that active chlorophyllase is localized in this region of the gel, whereas several inactive proteins show electrophoretic mobility values >0.4 (see fig.1). The isolated gel parts were ground thoroughly with 2 g sea sand and then extracted twice with ~50 ml 'Tris–EDTA'. The combined extracts were concentrated on an Amicon YM 10 filter to ~5 ml final vol.

(d) *Gel filtration:* Some residual inactive protein with relatively low M_r was eliminated by passing the light-yellow preparation through a Sephadex G-100 column, equilibrated with 'Tris–EDTA' (fig.2). The column was ~60 cm, long, 16 mm diam., at 10°C. The active fractions (each of 5 ml) were combined (see section 3) and once more concentrated by ultra-filtration over a YM-10 filter.

(e) *Adsorption to immobilized concanavalin-A:* The proteins were washed, on a YM-10 filter, with 0.02 M Tris–HCl buffer (pH 7.4) containing 5×10^{-4} M EDTA and 0.5 M NaCl. The protein solution in this buffer was mixed with 5 ml thrice-prewashed concanavalin-A–Sepharose 4B suspension and incubated for 30 min at room temperature. The gel was then separated from the supernatant and washed once with the 'Tris–EDTA'–NaCl buffer. Gel-bound chlorophyllase was released by adding the same buffer to which 0.2 M α-methyl-D-mannoside had been added. The mixture was left standing for 30 min at room temperature and thereafter the supernatant was separated from the gel. Both collected supernatants were concentrated by ultrafiltration, washed several times with 'Tris–EDTA' and reconcentrated. The final amount of recovered active enzyme was ~2–3 mg; the amount of 'inactive' enzyme (see section 3) was generally 4–5-times higher.

2.4. *Analytical polyacrylamide gel electrophoresis*

SDS gel electrophoresis was carried out according to [7] in 0.1% SDS on gels 6 cm long and 5 mm diam.; polyacrylamide was 7%. Proteins were dissociated with 1% SDS, in the presence of 1% β-mercapto-ethanol, for 30 min at room temperature, or for 10 min at 56°C followed by 10 min incubation at room temperature.

Triton electrophoresis was carried out in the same way as preparative electrophoresis, with 6 cm long gels of 5 mm diam. In both cases bromophenol blue was used as a tracking dye. The gels were fixed and then stained with either Coomassie brilliant blue R or with periodic acid–Schiff staining reagent [6].

2.5. *Chlorophyllase activity*

This was assayed as in [8]; the substrate chlorophyll was dissolved in acetone instead of methanol [9]. Maximum actone concentration in the reaction mixture was 10%.

2.6. *Protein concentration*

This was estimated either from 280 nm adsorption or by means of the Bio-Rad microassay, which is based on colour changes of Coomassie brilliant blue [10].

3. Results and discussion

3.1. *Size heterogeneity of chlorophyllase*

Polyacrylamide gel electrophoresis of chlorophyllase in the presence of 0.1% Triton leads to the localization of the enzyme activity in 2 or 3 bands instead of 1 in the upper part of the gel; activity correlates with distinct protein bands (fig.1). Similar results have

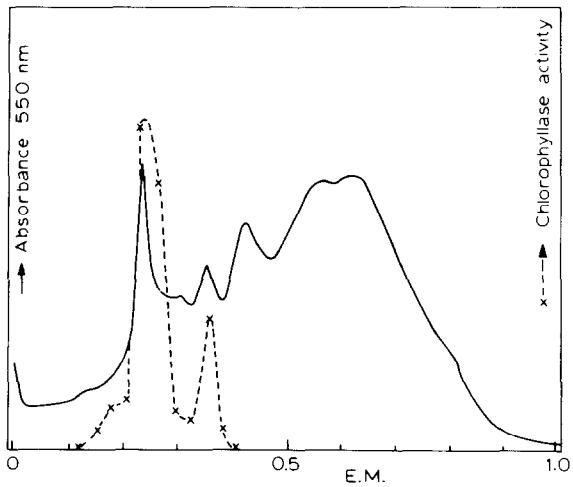

Fig.1. Triton gel electrophoresis pattern of an aqueous extract of acetone-precipitated *Phaeodactylum* membranes: (——) Coomassie blue absorbance; (–x–) chlorophyllase activity (% added chlorophyll converted into chlorophyllide); (E.M.) electrophoretic mobility.

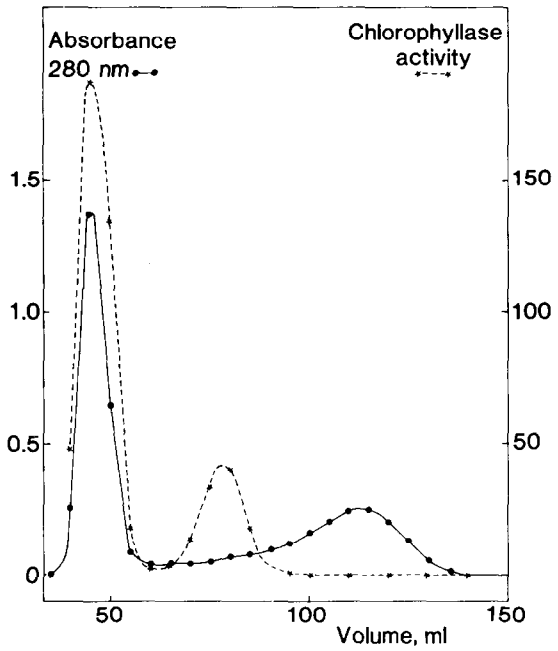

Fig.2. Fractionation of chlorophyllase by Sephadex G-100 gel filtration. Sample preparation: see text. Chlorophyllase activity (% added chlorophyll converted into chlorophyllide) was calculated for 5 μl of each 5 ml fraction.

Table 1
Influence of concanavalin A–Sepharose treatment on chlorophyllase activity

Preparation	Chlorophyllase activity (%)
(a) Untreated chl-ase solution (control)	100
(b) Chl-ase solution incubated with con A–Sepharose	5
(c) Con A–Sepharose + adsorbed chl-ase	15
(d) Chl-ase released from con A–Sepharose by α-methyl-D-mannoside	75

Abbreviations: Chl-ase, chlorophyllase; con A, concanavalin A

Procedure: 1 ml purified chl-ase solution, containing 30 μg protein, was incubated for 1 h at room temperature with ~0.5 ml twice-prewashed con A–Sepharose. The total volume was adjusted to 10 ml with 0.02 M Tris–HCl buffer (pH 7.4) containing 5×10^{-4} M EDTA and 0.5 M NaCl. The control (a) consisted of the same components without con A–Sepharose and was incubated in the same way. After incubation, the con A–Sepharose was separated from the supernatant by centrifugation for 15 min at 10 000 × *g*. (b) The con A–Sepharose was again diluted with the 'Tris–EDTA'–NaCl buffer to 10 ml (c). α-Methyl-D-mannoside was added to part of this solution to give 0.2 M final conc. and the mixture was left to stand for 45 min at room temperature. The con A–Sepharose was again separated from the supernatant (d). The solutions (a–d) were assayed for chlorophyllase activity

been reported for intrinsic membrane proteins [11,12] and for glycoproteins [13–15]. The effect has been ascribed to oligomer formation at low detergent concentrations.

Upon gel filtration of the combined active proteins obtained with Triton–polyacrylamide gel electrophoresis, chlorophyllase is separated into 2 fractions (fig.2). The first active fractions, immediately after the void volume, contains most of the protein; the second active fraction was not clearly correlated with a protein maximum. Although there was a higher chlorophyllase activity/protein unit in the second fraction, no difference could be detected in the behaviour of the enzymes in either fraction.

The results of both Triton-gel electrophoresis and Sephadex-gel filtration fit in with the earlier conclusion [16,17–20] that chlorophyllase in aqueous solutions tends to aggregate. Alternatively, the observed behaviour of the enzyme could be caused by polydispersity of the preparation (cf. [21]).

3.2. *Carbohydrate in chlorophyllase*

After electrophoresis, polyacrylamide gels loaded with purified chlorophyllase showed periodic acid–Schiff-staining bands which coincide with enzyme activity. This suggests that chlorophyllase contains carbohydrates.

Incubation of a chlorophyllase solution with concanavalin A–Sepharose resulted in binding of the active enzyme. Chlorophyllase in its bound form was, for the most part, inactivated. The active enzyme could be released from the Sepharose by suspending the gel in buffer containing 0.2 M α-methyl-D-mannoside (table 1). From these results it was concluded that:

(1) Chlorophyllase is a glycoprotein, containing a carbohydrate residue with concanavalin A-binding specificity [22–25];
(2) The active site of chlorophyllase reacts directly with concanavalin A or becomes masked by concanavalin A bound to an adjacent carbohydrate moiety of the enzyme molecule.

When a purified enzyme preparation was treated with concanavalin A–Sepharose, ~20% of the protein was bound and subsequently released by 0.2 M α-methyl-D-mannoside. This fraction exhibited high

chlorophyllase activity. About 80% of the enzyme preparation was not bound and was only slightly active. The bound fraction was, per protein unit, 100–150-times more active than the unbound fraction. The latter fraction will be called 'inactive'.

3.3. *Polypeptide subunit composition of chlorophyllase*

Both active and 'inactive' fractions, separated by concanavalin A–Sepharose adsorption, formed similar (glyco)protein bands upon Triton-gel electrophoresis. The polypeptide composition of these fractions was determined by SDS–polyacrylamide gel electrophoresis. The gels were stained for proteins with Coomassie blue and for glycoproteins (carbohydrates) with periodic acid–Schiff reagent. In active as well as in 'inactive' fractions, 3 or 4 principal protein bands, stained by Coomassie blue, were visible, in addition to minor bands (fig.3).

Although there were quantitative variations, no significant differences were observed in the qualitative polypeptide composition of active and 'inactive' protein fractions.

When the periodic acid–Schiff staining method was used, both active and 'inactive' fractions showed 2 major bands and 1 minor band. The latter, with electrophoretic mobility of 0.6 in our experiments, coincides with an active 38 000 M_r chlorophyllase subunit [3,19,20,26]. Only in the active fractions did a band show up at electrophoretic mobility 0.25 (fig.4). This band was also detected in chlorophyllase preparations that had not been treated with concanavalin A–Sepharose and α-methyl-D-mannoside. This excludes the possibility that the band is due to one of these reagents.

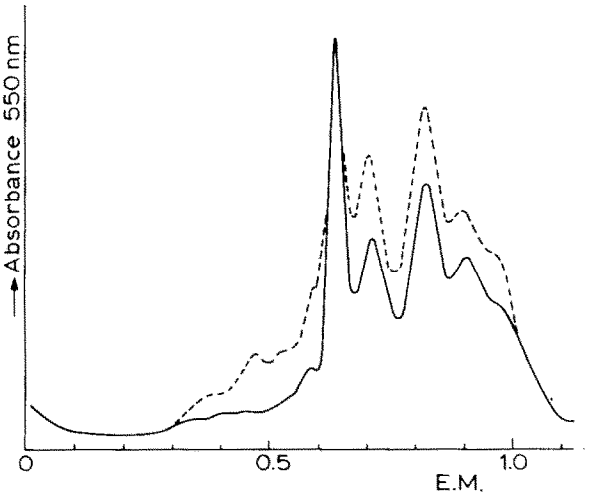

Fig.3. SDS gel electrophoresis patterns of purified chlorophyllase treated with concanavalin A–Sepharose; gels were stained with Coomassie blue; (– –) concanavalin A-nonreactive, enzymatically little active fraction; (——) concanavalin A-reactive, enzymatically highly active fraction; (E.M.) electrophoretic mobility.

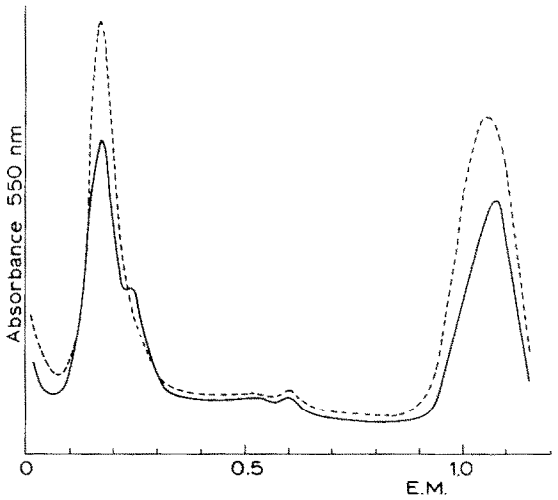

Fig.4. SDS gel electrophoresis patterns of purified chlorophyllase treated with concanavalin A–Sepharose; gels were stained with periodic acid–Schiff reagent; (– – –) concanavalin A-nonreactive, enzymatically little active fraction; (——) concanavalin A-reactive, enzymatically highly active fraction; (E.M.) electrophoretic mobility.

If the enzymatically 'inactive', concanavalin A-nonreactive fraction consisted of contaminating proteins, the polypeptide pattern would differ from that of active chlorophyllase. The fact that the patterns of both the active and 'inactive' fractions are similar (fig.3,4) suggests that both fractions contain almost the same proteins. This protein must consist of several subunits, 2 or 3 of them having a carbohydrate moiety. The active fraction contains 1 extra glycopeptide subunit (electrophoretic mobility 0.25 in our experiments). Possibly, this special carbohydrate moiety is essential for enzymatic activity as well as for concanavalin A-reactivity of the active form of chlorophyllase.

Contrary to Triton X-100, SDS seriously inhibits the enzymatic activity of chlorophyllase, presumably through its influence on enzyme conformation. Nevertheless, after SDS electrophoresis some residual

enzymatic activity can be measured in a 38 000 M_r polypeptide (electrophoretic mobility of 0.60 in the circumstances of our experiments). (see [3]). At the site of this slightly active polypeptide a comparatively slightly stained band shows up after periodic acid–Schiff staining (fig.4). Such a band, but without enzymatic activity, is also found upon SDS electrophoresis of 'inactive' enzyme. The results lead us to the hypothesis that for chlorophyllase activity both a 38 000 M_r polypeptide, in its 'native' conformation, and a special carbohydrate-containing moiety are required. The function of the other (glyco)polypeptides that show up upon SDS gel electrophoresis is still unknown (cf. [27]).

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