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Chlorophyll decomposition in *Skeletonema costatum:* a problem in chlorophyll determination of water samples

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ABSTRACT: Chlorophyll decomposition during the process of chlorophyll determination currently used for aquatic samples was examined with cultured phytoplankton. Among the phytoplankton tested, chlorophyll *a* in *Skeletonema costatum* isolated from Tokyo Bay was found to be extremely unstable. Filtration of cell suspension with gentle suction caused chlorophyll *a* decomposition mainly to phaeophorbide *a*. Decomposition of over 40 % of total chlorophyll *a* occurred resulting an erroneous estimation of phaeopigment proportion in the samples. The decomposition was a result of a high activity of chlorophyllase and acidic material(s), probably organic acid(s), accumulated in the cells. Since the decomposition was found to be initiated by chloroplast damage, we tried to use a hypertonic medium for stabilization of chloroplast structure. Washing of samples with a hypertonic medium (0.4 M mannitol plus 1 mM $MgCl_2$ and 3.15 % NaCl) just before finish of filtration was found to give 89 % recovery of chlorophyll *a*. However, the effect was not very reproducible.

INTRODUCTION

In ecological studies of aquatic areas, chlorophyll distribution has been regarded as an important index for estimation of phytoplankton mass and capacity of primary production. The method for quantitative analysis of this chemical is well-established. Manuals on aquatic ecology (e.g. Vollenweider 1969, Strickland & Parsons 1972) have recommended a simple method of chlorophyll measurement for this purpose. The method basically consists of (i) collection of phytoplankton cells on filter paper with gentle suction, (ii) acetone extraction with the aid of mechanical disruption or soaking in cold acetone, and (iii) spectrophotometric or fluorometric measurement. The method currently used has been slightly modified by different research groups to avoid chlorophyll decomposition during the measuring process, e.g. filtration of the samples after $MgCO_3$ addition to protect from phaeopigment formation (cf. Yanagi & Koyama 1971, Strickland & Parsons 1972), extraction with absolute acetone to kill chlorophyllase activity (cf. Jeffrey 1974). However, the basic procedure is the same in collection of test material from waters and in extraction of chlorophyll without heating. These methods for extraction have been believed to be safe from chlorophyll decomposition. The pigment composition in samples obtained by these methods has been used as an index of the physiological state of phytoplankton (Yanagi & Koyama 1971, Jensen & Sakshaug 1973, Jeffrey 1974, Gieskes et al. 1978, Hallegraeff 1981) and grazing action of zooplankton (Jeffrey 1974, Shuman & Lorenzen 1975, Hallegaeff 1981).

Chlorophylls *in vitro* are not necessarily stable. Photobleaching of chlorophylls in broken cells (Moreth & Yentsch 1970), or chlorophyllase action (Barett & Jeffrey 1964, 1971) are examples. As a first step in the chemical study of chlorophylls in marine samples, we re-examined the method currently used for chlorophyll determination and found that chlorophyll *a* in *Skeletonema costatum* isolated from Tokyo Bay is considerably unstable; a marked decomposition of chlorophyll *a* to phaeophorbide *a* via chlorophyllide *a* occurred during the filtration process using gentle suction. In this paper, we report the results obtained from analysis of this chlorophyll decomposition and discuss a possible way of protection from such artificial decomposition.

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MATERIALS AND METHODS

Algal cultures. The tests were made with the marine diatoms *Chaetoceros socialis*, isolated from Sagami Bay, and *Skeletonema costatum*, isolated from Tokyo Bay and with the dinoflagellate *Prorocentrum minimum*, isolated from Tokyo Bay; the latter 2 were originally isolated by Dr T Ishimaru (Ocean Research Institute, University of Tokyo) and kindly supplied by him. Cells were grown in the f medium of Guillard & Ryther (1962) at half nutrient strength at 18 °C. Air containing 0.5 % CO_2 was continuously supplied, as was illumination with fluorescent light (6.0 Wm^{-2} for *C. socialis* and *S. costatum*, and 8.0 Wm^{-2} for *P. minimum*). Cells at the late exponential growth phase were used for all experiments.

Cell harvesting and chlorophyll extraction. Cells were collected from cultures in 2 ways: (i) cell suspensions were filtered through a Whatman GF/C filter paper with gentle suction to collect cells on the filter paper (we will call this treatment 'Filtration'), and (ii) cell suspensions were centrifuged at 2000 × g for 5 min, and cells collected as pellets ('Centrifugation'). Chlorophyll extraction with acetone was also made in 2 ways: (i) repeated extraction at 65 °C for 2 min ('Heat') and (ii) grinding with acetone in a glass mortar below 25 °C ('Grinding'). After removing cell and glass fiber debris, the acetone extracts were used for spectrophotometry and chromatographic analysis.

Chromatographic analysis. Extracts were concentrated with a flash evaporator at 25 °C in the dark. Evaporation was generally completed within 5 min. For separation of chlorophyll *a* and phaeophytin *a*, paper chromatography with a solvent system of petroleum ether containing 1.35 % *n*-propanol was used. Rf values for chlorophyll *a* and phaeophytin *a* were 0.75 and 0.90, respectively. Those of chlorophyllide *a* and chlorophyll *c* were as low as 0.1. Thin layer chromatography of reverse phase was used for phytol-free pigments. Pigments were separated by a Merk MCK_{18} plate with a solvent system of methanol/acetone/water (20 : 4 : 3 in volume). Rf values of chlorophyllide *a*, phaeophorbide *a* and chlorophyll *c* were 0.7, 0.4 and 0.8, respectively; chlorophyll *a* and phaeophytin *a* remained at the starting point. Each pigment was quantitatively eluted from chromatograms by aqueous acetone (80 %).

Spectrophotometry. Absorption spectra of pigment samples in acetone were measured by a Shimadzu UV 200 spectrophotometer at room temperature. Pigment concentration was calculated by using absorption coefficients reported by Mackinney (1941) for chlorophyll *a* and phaeophytin *a*, and by Jeffrey (1972) for chlorophyll *c*. For estimation of chlorophyllide *a* and phaeophorbide *a*, the values for chlorophyll *a* and phaeophytin *a* were used, respectively.

RESULTS AND DISCUSSION

Chlorophyll decomposition during extraction

The Grinding treatment is a likely source of chlorophyll decomposition. We examined this possibility first, with extracts from 3 phytoplankton species. Cells were collected by Centrifugation, and extraction was made with acetone of various concentrations. Cells were ground with a small piece of GF/C filter paper for 60 s at room temperature. Results are presented in Table 1. In each case, the experiment was repeated 3 times. Grinding for a short time did not cause any appreciable decomposition in the case of *Prorocentrum minimum*; a small amount of decomposed components was found in *Chaetoceros socialis*. The Grinding treatment seems to be safe in these phytoplankton. However, a large amount of chlorophyllide *a* and phaeophorbide *a* was found in the extract of *Skeletonema costatum*. In the extreme

Table 1. Chlorophyll *a* decomposition during grinding extraction in various acetone concentrations. Cells were collected by Centrifugation, and chlorophylls were extracted by Grinding at room temperature. For details of experiments, see text

Organism	Acetone concentration (%)	Relative content (%) of		
		Chlorophyll *a*	Chlorophyllide *a*	Phaeophorbide *a*
Skeletonema costatum	100	73.4 ± 10.4	15.9 ± 1.5	10.7 ± 2.3
	80	52.8 ± 6.4	46.9 ± 13.5	0.3 ± 0.03
	50	35.6 ± 4.3	59.8 ± 5.9	4.6 ± 0.4
Chaetoceros socialis	100	98.7 ± 0.7	1.3 ± 0.2	0
	80	99.2 ± 2.8	0.8 ± 0.3	0
	50	98.2 ± 1.2	1.8 ± 0.6	0
Prorocentrum minimum	100	100.0	0	0
	80	100.0	0	0
	50	100.0	0	0

case, almost 65 % of chlorophyll *a* was found as decomposition products. The major product was chlorophyllide *a*. Phaeophytin *a* was insignificant. Since the occurrence of decomposition products depended on the acetone concentration, such pigments are probably not present in the original samples, but are formed by chlorophyllase action during Grinding. Jeffrey (1974) and Riper et al. (1979) reported that chlorophyll extraction with absolute acetone can kill chlorophyllase in the sample. However, chlorophyllide *a* and phaeophorbide *a* occurred even in extraction with absolute acetone. The killing action is not necessarily sufficient in this case. Occurrence of chlorophyllide *a* in extracts from *C. socialis* also seems to depend on the acetone concentration. It may again be an artificial product due to chlorophyllase action.

As shown in Table 2, extracts by the Heat treatment contained very small amount of chlorophyllide *a*, while a large amount of decomposed pigments occurred in the extracts by Grinding. Decomposition also depended on the temperature during Grinding. Cell disruption without killing chlorophyllase must be a primary cause of chlorophyll decomposition. The cell disruption probably first induced chlorophyllase activation; phaeopigment formation will then occur. To confirm this idea, we examined the effect of cell disruption by sonication. A dense cell suspension (equivalent to 33 µg chlorophyll *a* ml^{-1}) was sonicated for 1 min at 0 °C. Pigments were extracted from the sonicates by Heat treatment. Table 3 compares pigment compositions before and after sonication. Occcurrence of decomposed pigments was found only in the samples after sonication as expected.

Chlorophyll decomposition during filtration

In most cases of chlorophyll determination of water samples, phytoplankton cells in water are first collected on a glass-fiber or membrane filter with gentle suction. During filtration, cells placed on the filter will suffer from mechanical destruction due to being pressed onto the filter surface. Such an effect will be especially strong on drying the filter after filtration. This effect was examined for *Skeletanema costatum* by comparing pigment compositions in cell samples collected by Filtration and by Centrifugation. Pigments were extracted by Heat, which is safe from chlorophyll decomposition (cf. Table 2 & 3). Table 4 clearly indicates that Filtration also causes chlorophyll decomposition. The cell sample collected by Centrifugation contained only a small amount of decomposed pigment (chlorophyllide *a*, 5 %), while more than 40 % of chlorophyll *a* was decomposed in the samples collected by Filtration. Filtration was finished within 15 s in this case, and extraction made immediately afterwards. Decomposition progressed further when the samples were left at room temperature in the dark. After 20 min, the chlorophyll *a* content was reduced to 16 %. An $MgCO_3$ layer placed on the filter surface did not improve the result (data not shown).

Results indicate that chlorophyll decomposition is more marked in Filtration than in the Grinding process. The main product was phaeophorbide *a*, similar to the case of cell sonication (Table 3), but different from the case of Grinding (Tables 1 & 2) where the main product was chlorophyllide *a*. The decomposition products seem to depend on the cell density during cell

Table 2. *Skeletonema costatum*. Chlorophyll *a* decomposition during Heat and Grinding extraction methods. Cells were collected by Centrifugation, and chlorophylls extracted by hot acetone (65 °C, 2 min) for Heat, and for Grinding, by grinding in a glass mortar with absolute acetone at respective temperature. For details of experiments, see text

Method	Relative content (%) of		
	Chlorophyll *a*	Chlorophyllide *a*	Phaeophorbide *a*
Heat	95.2 ± 7.8	4.9 ± 0.4	0
Grinding, 0 °C	73.4 ± 10.4	15.9 ± 1.5	10.7 ± 2.3
Grinding, 30 °C	42.9 ± 10.4	21.7 ± 4.4	35.3 ± 17.5

Table 3. *Skeletonema costatum*. Chlorophyll *a* decomposition and cell destruction. Cells were collected by Centrifugation and resuspended in sea water at high cell density (33 µg chl *a* ml^{-1}). Chlorophylls were extracted with absolute acetone by Heat without sonication for 'Intact', and after sonication for 1 min at 0 °C for 'Broken'. For details of experiments, see text

Cell condition	Relative content (%) of			
	Chlorophyll *a*	Chlorophyllide *a*	Phaeophytin *a*	Phaeophorbide *a*
Intact	100	Trace	0	Trace
Broken	20 ± 1.7	6.8 ± 0.86	9.0 ± 0.81	65 ± 16

Table 4. *Skeletonema costatum*. Chlorophyll *a* decomposition and cell collection. For Centrifugation, cells were collected by Centrifugation, and chlorophylls were extracted by Heat. For Filtration, cells were collected on the GF/C by filtration with gentle suction, and chlorophylls extracted with absolute acetone by Heat either immediatedly after filtration (Filtration, 0 min), or after 20 min at room temperature in the dark (Filtration, 20 min). For details of experiments, see text

Collection method	Relative content (%) of			
	Chlorophyll *a*	Chlorophyllide *a*	Phaeophytin *a*	Phaeophorbide *a*
Centrifugation	95.0 ± 1.9	5.0 ± 1.5	0	0
Filtration, 0 min	57.0 ± 4.7	11.8 ± 5.0	2.2 ± 1.2	29.0 ± 3.3
Filtration, 20 min	15.8 ± 7.0	0	7.7 ± 2.6	76.5 ± 2.3

disruption; at high cell density, phaeophorbide *a* is mainly formed, and at low density, chlorophyllide *a*. Sonic treatment was made at high cell density. Cell breakage during Filtration must occur after the cells have been pressed on to the filter, and are thus at very high density. On the other hand, cells were disrupted at low density in the Grinding treatment. The relation between phaeopigment formation and cell density suggests that phaeopigment formation is induced by an acidic material accumulated in the cells, probably organic acid(s). In Grinding, such acidic material would be diluted by the medium immediately after cell breakage.

Occurrence of phaeophorbide *a* but not phaeophytin *a* indicates that phaeopigment formation occurs after chlorophyllide *a* is formed. Since chlorophyllase is present in the chloroplasts (Ardao & Vennesland 1960), chloroplast breakage, and thus disorganization of thylakoid, will induce activation of chlorophyllase. Chlorophyllase will attack chlorophyll-protein complexes in the thylakoid membranes and form chlorophyllide *a* which is no longer bound tightly to the protein complexes. The chlorophyllide *a* released will then suffer from the acidic environment caused by acidic material present at the outside of chloroplasts, and will be converted to phaeophorbide *a*. Therefore, chlorophyll decomposition in *Skeletonema costatum* may be characterized by an active chlorophyllase and by an accumulation of acidic material in the cells.

The phaeopigment proportion of samples is an important index for aquatic ecology. Chlorophyll decomposition observed for Filtration is a serious problem, if the phytoplankton in the water samples has a similar nature to *Skeletonema costatum*. However, in Grinding, only a small amount of phaeopigment was formed, so this process is fairly safe in this sense. A high chlorophyllase activity has been found in many diatoms (Barret & Jeffrey 1964, 1971). Phaeopigment formation observed here may not be specific to *S. costatum*.

Protection of chlorophyll decomposition by hypertonic medium

Chloroplasts may be kept unbroken even *in vitro* when the cells are broken in a hypertonic or isotonic medium such as those used for isolation of intact chloroplasts from higher plants (Cockburn et al. 1968). With this idea, we examined the effect of hypertonic treatment on chlorophyll decomposition during Filtration. It is not practical to make the whole sample hypertonic by adding sugar or polyalcohol before filtration. We tried to wash the samples on the filter

Table 5. *Skeletonema costatum*. Effect of hypertonic washing on chlorophyll *a* decomposition. Cells were collected by Filtration with or without washing by the respective hypertonic medium, and chlorophylls were extracted with absolute acetone by Heat. Control was by Centrifugation cell collection and Heat extraction. For details of experiments, see text

Treatment	Relative content (%) of			
	Chlorophyll *a*	Chlorophyllide *a*	Phaeophytin *a*	Phaeophorbide *a*
Filtration, 0 min				
without washing	66.7 ± 4.7	14.0 ± 0.7	9.3 ± 1.0	10.0 ± 1.3
washing, Medium I	75.3 ± 9.0	17.5 ± 0.6	0	7.1 ± 1.3
washing, Medium II	90.0 ± 2.7	4.3 ± 0.9	0	4.9 ± 1.0
Filtration, 5 min				
without washing	37.7 ± 8.5	7.9 ± 1.9	21.1 ± 1.9	33.1 ± 2.0
washing, Medium II	79.4 ± 10.2	6.4 ± 1.6	0	14.0 ± 1.6
Control	94.0 ± 0.7	3.7 ± 1.0	0	2.5 ± 0.9

paper with the hypertonic medium just before the finish of filtration. As the washing medium, 0.4 M mannitol solution (I) or 0.4 M mannitol plus 1 mM $MgCl_2$ and 3.5 % NaCl solution (II) was tested. Results are shown in Table 5. Medium I was not very effective for protecting the sample against chlorophyll decomposition. However, washing with Medium II markedly reduced chlorophyll decomposition; only 5 % of chlorophyll *a* was decomposed to phaeopigment. The effect persisted for at least 5 min after filtration. Results confirm the above idea and indicate that hypertonic washing is one method of protecting samples from chlorophyll decomposition.

However, the effect is not necessarily perfect and reproducible. The medium may be too simple for the purpose. A more sophisticated composition will be required, similar to the case for isolation of Class I type chloroplasts from higher plants (cf. Cockburn et al. 1968, Reeves & Hall 1980). Another problem is the occurrence of cell breakage during filtration; it must occur for the cells placed on the filter surface, and may reduce the reproducibility of the protecting effect. Further improvement is necessary before putting the method in practice.

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