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Development of a Rapid Isolation Procedure for Coccolith Ultrafine Particles Produced by Coccolithophorid Algae

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ABSTRACT

A rapid procedure for effective purification of large quantities of coccolith ultrafine particles from marine algae is reported. Coccoliths are detached from cells by optimized sonication in the presence of 50 m*M* $NaHCO_3$. Contaminating cell debris is then removed from coccoliths by cycles of washing and floatation. Coccolith particles were purified from *Emiliania huxleyi* and *Pleurochrysis carterae*. The surface area of these particles is three to five times greater than synthetic calcite particles. Glucose oxidase and uricase have been immobilized onto purified coccolith ultrafine particles to illustrate their potential as a support material for biotechnological application.

Index Entries: Coccolithophorid algae; coccolith; calcite ultrafine particles; enzyme immobilization.

INTRODUCTION

Both oceanic primary production and calcium carbonate deposition by marine planktonic algae are of global importance in relation to the carbon cycle. The Coccolithophorid algae *Prymnesiophyceae* produce extracellular

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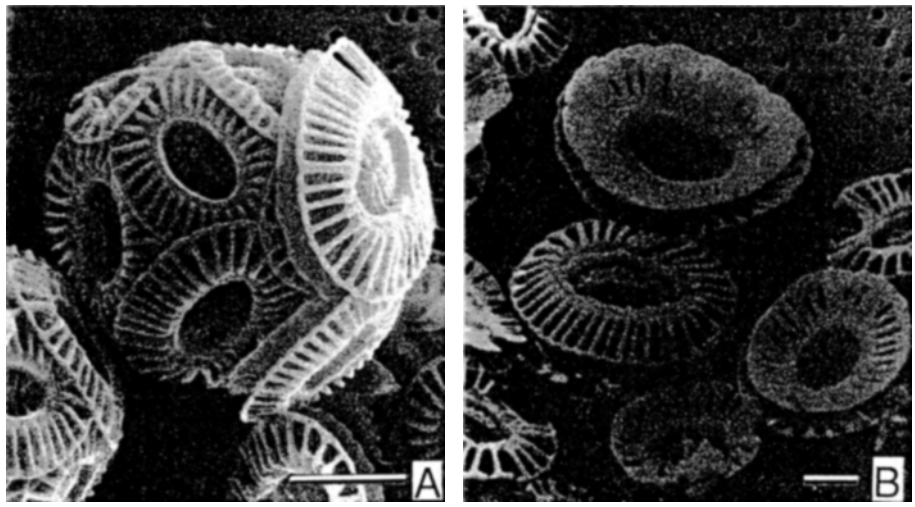

Fig. 1. Scanning electron micrographs of *Emiliania huxleyi* cell (A) and isolated coccolith particles (B). Bars indicate 2 μm (A) and 1 μm (B).

plates of $CaCO_3$ formed into species-specific shapes. Mineralization by coccolithophorid algae differs from that of many organisms in that a complex mineral structure is produced and precisely assembled within a single Golgi vesicle (*1–4*). The secreted structures are known as coccoliths, and once formed, they are extruded through the cell membrane and encase the cell.

Heterococcoliths, one of the basic types of coccoliths, are complex-shaped crystals that are made up from a variety of elaborate scales and ornate spines. In *Emiliania huxleyi,* they are composed of about 30 radially arranged units of crystalline $CaCO_3$; each segment of a coccolith consists of an upper and a lower element with a connecting tube between them (Fig. 1). *P. carterae* coccoliths consist of two functional parts. Eleven to 19 segments form a ring, and additional segments act as a clasp to connect those ring segments rather like a chain (Fig. 2).

Coccoliths were discovered more than a hundred years ago. Photosynthesis of coccolithophorid algae and the mechanism by which coccolith particles are produced have been studied (*5–8*), but biotechnological applications of these composite materials have not been investigated. Present methods for isolating coccolith are complicated and not suited to treating large cell counts (*9*). A simplified procedure has been developed that can be used for large-scale purification. Coccoliths required for indusrial application can therefore be supplied readily.

The previous method needs more than six steps, many chemicals, and to be centrifuged by high gravity. Therefore, increasing sample volume is difficult. However, the authors' method needs three steps and only one chemical, and the separation method by floatation of coccolith

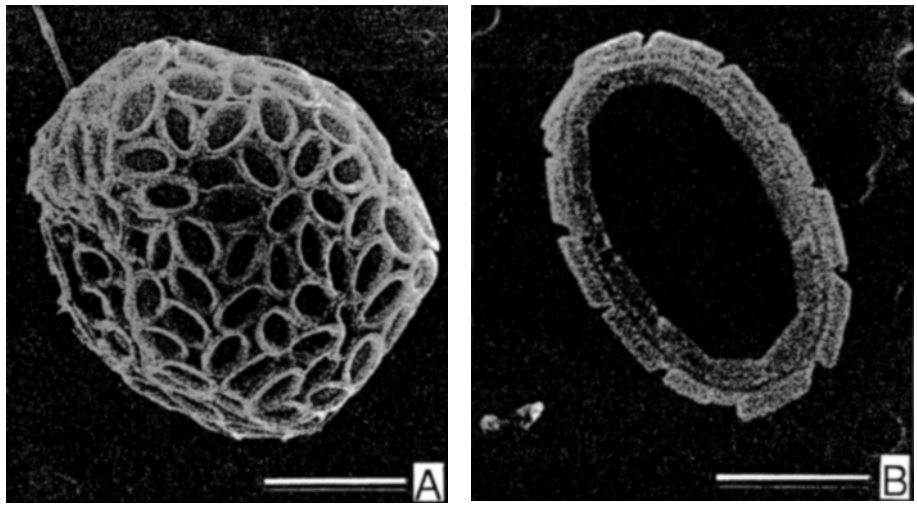

Fig. 2. Scanning electron micrographs of *Pleurochrysis carterae* cell (A) and isolated coccolith particles (B). Bars indicate 5 μm (A) and 1 μm (B).

particles is easy to increase sample volume. Compared with the previous methods, this method is particularly convenient. If adopted, the method could lead to the production of a great deal of coccolith for a variety of industrial uses. By noting that coccoliths are composed of distinctive ultrafine particles by calcite crystals, an attempt was made to use them as immobilizing supports. This article describes enzyme immobilization using coccolith ultrafine particles isolated from *Emiliania huxleyi* and *Pleurochrysis carterae*.

MATERIAL AND METHODS

Reagents

Glucose oxidase was supplied by Amano Co., Ltd. (Nagoya, Japan), and Uricase (Oriental Yeast Co., Ltd., Tokyo, Japan) was used. Other reagents were commercially available analytical- or laboratory-grade materials.

Cell Strains and Culture Conditions

Coccolithophorid algae *Pleurochrysis carterae* and *Emiliania huxleyi* were obtained from E. W. de Jong (Department of Biochemistry, State University of Leiden). Eppley's medium (*10*), a nutrient-enriched natural sea water, was used. The algae were cultivated aerobically at 20°C, and a 16-h light, 8-h dark cycle was maintained. *E. huxleyi* and *P. carterae* were cultivated at light intensities of 90 $\mu E\ m^{-2}\ s^{-1}$ and 150 $\mu E\ m^{-2}\ s^{-1}$, respectively.

Sonication Treatment

A beaker containing 1 L of cell suspension at a concentration of 0.01–0.6% (dry cell wt/vol) was placed in a sonication bath (UT-12X Shinmeidai-kogyo Co. Tokyo, Japan) connected to an Ultrasonic generator (U0150FX, frequency 27KHz, maximum power 150 W, Shinmeidai-kogyo Co., Tokyo, Japan). Sonication power was fixed at 30 W in this study. Sonication treatment was performed at various times. The number of coccoliths and viable cells was determined by light microscopy.

Immobilization of Enzymes on Coccolith Ultrafine Particles

Purified coccoliths were washed in 0.1*M* H_3BO_4-NaOH buffer (pH 9.5) and suspended in 1 mL of the buffer, in which 1 mg of each of the enzymes was dissolved. It was then left to stand at 4°C with stirring for 12 h. The total amount of protein in the enzyme solution was determined by Lowry's method (*11*) before and after immobilization of the enzymes. The amount of enzyme immobilized on the coccolith ultrafine particles was calculated from the difference in amounts of protein of enzyme solutions.

Determination of Enzyme Activity

For determination of uricase (urate oxidase; EC1.7.3.3) activity, 1 mg of coccolith particles and synthetic calcite, coupled with uricase, was added to 3.1 mL of borate buffer (0.1*M* and pH 9.5) containing 40 μg urate. After the reaction mixture was incubated at 37°C for 10 min, 0.02 mL of 10*N* NaOH was added to stop the enzyme reaction. The reaction mixture was centrifuged (10,000 rpm, 2 min). The resulting supernatant was measured with the spectrophotometer at 293 nm.

For determination of glucose oxidase (EC1.1.3.4) activity, 1 mg of coccolith particles and a synthetic calcite coupling with glucose oxidase were added to 3 mL of phosphate buffer (0.1*M*, pH 8.0 and O_2 saturated) containing 66 μg *o*-dianisidine-HCl, 50 μg D-glucose, and 20 μg peroxidase (0.006 U). After the reaction mixture was incubated at 25°C for 5 min, 0.02 mL of 10*N* NaOH was added to stop the enzyme reaction. The reaction mixture was centrifuged (10,000 rpm, 2 min), and absorbance of the resulting supernatant was measured with a spectrophotometer at 436 nm.

Analytical Procedures

The specific surface areas of coccoliths and of synthetic calcite were determined by the nitrogen gas absorption-desorption method, using a P-700 specific surface analysis system (Shibata Kagaku-Kiki Co., Ltd., Tokyo, Japan). The amount of calcium carbonate and organic matter was determined by thermal analysis (TG-DTA) using TG-30M (Shimadzu Corp., Kyoto, Japan). The rate of heating of the samples was kept at 10°C

min^{-1}, and a 10-mg sample was analyzed, using air atmosphere. Mineral compositions of coccoliths were determined by X-ray fluorescence spectrometry using 3080E2 (Rigaku Denki Co., Tokyo, Japan). X-ray diffraction analysis was carried out using a Rigaku X-ray powder diffractometer (Ru-200B, Rigaku Denki Co., Tokyo, Japan) with Cu Kaipha radiation.

Scanning Electron Microscopic Analysis

After filtering a sample suspension under low pressure onto a polycarbonate filter (pore size 0.2 μm, diameter 25 mm, Nihon-Millipore Ltd., Tokyo, Japan), the sample was washed with 10 mL of NH_4HCO_3 and vacuum-dried in a desiccator. The dried filter was kept under vacuum (10^{-7} torr) for 3 h using a vacuum evaporator JEOL JFC-4X. Pt-Pd was vacuum-evaporated onto the filter. The sample was examined with a scanning electron microscope JOEL JSM-F15.

RESULTS AND DISCUSSION

Isolation of Ultrafine Calcite Particles from Coccolithophorid Algae

The isolation procedure was constructed from three parts;

1. Detachment of coccoliths from the cells;
2. Separation of coccoliths from cells; and
3. Removal of contaminating cell debris from coccoliths.

1. Detachment coccoliths from cells: Cells in a late-logarithmic phase were harvested by centrifugation (8000*g*, 10 min) and were washed with sea water. Six grams of *E. huxleyi* cells were suspended in 1 L of 50 m*M* $NaHCO_3$ and sonicated. Sonication was performed to detach coccoliths from the cells (frequency 27 kHz, power 30 W). The effect of sonication time on the number of detached coccoliths was examined, and the optimum sonication time was determined. In the case of *E. huxleyi*, the number of detached coccoliths gradually increased when the sonication time was increased from 30 s to 120 s (Fig. 1). However, when the sonication time was increased from 120 s to 200 s, only a slight increase occurred.
2. Separation of coccoliths from cells: The coccolith material was found to be contaminated with broken cell debris. This hampered further purification. When the sonicated cell suspension was allowed to stand for 12 h, detached coccoliths floated to the surface and were removed. The level of contamination with broken cell debris increased with sonication time owing to cell disruption. The effect of sonication time on the number

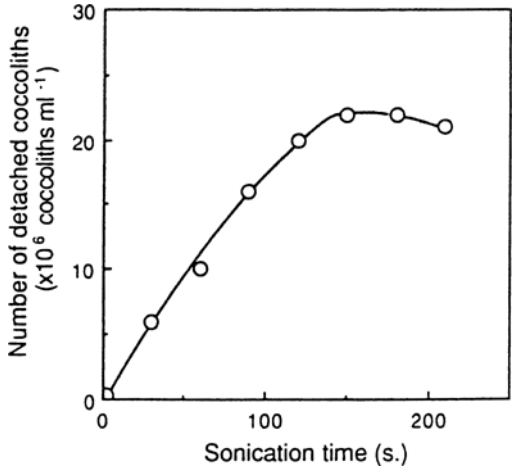

Fig. 3. Effect of sonication time on number of detached coccoliths from the cell of *Emiliania huxleyi*. Initial cell density of the treated culture was 1.8×10^6 cells/mL. Initial detached coccolith density in the treated culture before sonication was 8.1×10^6 coccoliths/mL.

of viable cells was therefore examined. For *E. huxleyi*, the number of viable cells decreased gradually beginning at sonication times as short as 60 s (Fig 3). Thus, contaminating cell debris increased gradually when sonication time was over 60 s.

3. Removal of contaminating cell debris: The resulting coccolith suspension was centrifuged (8000*g*, for 10 min). The pellet, containing coccoliths and broken cell debris, was suspended in 1 L of 50 m*M* $NaHCO_3$ and left to stand for 12 h with stirring. The resulting coccolith solution was centrifuged (8000*g*, for 10 min) and resuspended in 1 L of 50 m*M* $NaHCO_3$. The procedure (from the stirring to the centrifugation) was repeated twice. The pellet was then washed with 50 m*M* $NaHCO_3$ to give a purified coccolith sample.

White coccoliths (2.7 g) were obtained from 6-g cells of *E. huxleyi*. Using this method, 1 g of coccoliths was obtained from 6.6-g cells of *P. carterae*. Coccolith particle yields per dry cell wt from *E. huxleyi* and *P. carterae* were 45 and 17%, respectively. The isolated coccoliths were ultrafine particles that had a complex-shaped structure. The isolated coccolith particles were intact, and the diameter of particles isolated from *P. carterae* and *E. huxleyi* (Figs. 1, 2) ranged in size from 1–3 μm and from 2–4 μm, respectively (Table 1). There is no difference in shape of a coccolith attached to the cell surface and isolated coccoliths. Synthetic calcite particles had a simple cubic structure, whereas the coccolith particles consisted of intricate combined segments. The specific surface area of isolated coccolith

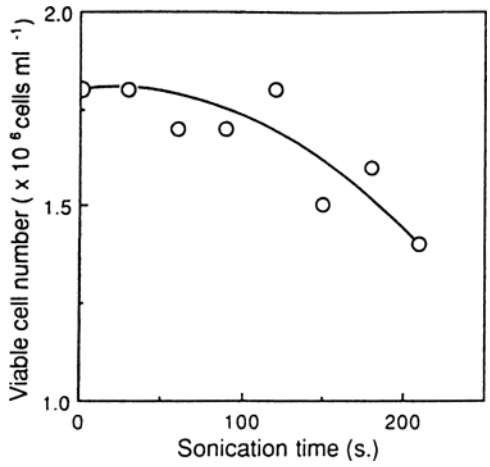

Fig. 4. Effect of sonication time on viable cell number of *Emiliania huxleyi*. Initial cell density of the treated culture was 1.8×10^6 cells/mL.

Table 1
Specific Surface Area and Diameter of the Isolated Coccolith Particles and Synthetic Calcite

Particles	Surface area, m^2/g	Diameter, μm
Isolated coccoliths from *E. huxleyi*	25.9	2–4
Isolated coccoliths from *P. carterae*	16.8	1–3
Synthetic calcite	5.9	2–3

was three to five times greater than that of synthetic calcite particles. The specific surface area of the coccolith particles isolated from *E. huxleyi* was more than 1.5 times that of the coccolith particles isolated from *P. carterae* (Table 1).

Chemical Composition of Coccolith Particles

The calcium carbonate content of *P. carterae* and *E. huxleyi* coccolith particles were 85.4 and 90.4%, respectively. X-ray diffraction analysis confirmed the crystalline form of calcium carbonate as calcite in both cases, and other crystalline forms of calcium carbonate were not observed. Coccolith particles isolated from *E. huxleyi* and *P. carterae* contained 3.5 and 4.5% of organic material, respectively. Metals other than calcite were present. In coccolith particles from *P. carterae*, Fe, Mg, Na, Sr, Si, and Zn were detected, whereas Fe, Mg, K, Na, Sr, and Si were found in coccolith particles isolated from *E. huxleyi*.

Table 2
Immobilization of Glucose Oxidase and Uricase
on Isolated Coccolith Particles and a Synthetic Calcite

Enzymes	Isolated coccoliths from *E. huxleyi*	Isolated coccoliths from *P. carterae*	Synthetic calcite
GOD			
Amount of immobilized enzyme (μg/mg)	27.5	16.3	5.8
Specific activity (U/mg)	4.8	2.0	1.2
Uricase			
Amount of immobilized enzyme (μg/mg)	25.8	15.5	6.5
Specific activity (U/mg)	6.0×10^{-2}	2.5×10^{-2}	1.5×10^{-2}

Enzyme Immobilization on Coccolith Particles

Glucose oxidase and uricase were immobilized on coccolith particles and synthetic calcite particles. Table 2 shows the amounts of adsorbed glucose oxidase and uricase. The amounts of enzymes adsorbed on the coccolith particle isolated from *P. carterae* and *E. huxleyi* were three and five times higher than those on synthetic calcite particles. The isolated coccolith particle was uniform in diameter and shape, and ultrafine particles had a high surface area. It is therefore quite likely that the differences in enzyme adsorption are the result of the different specific surface areas of each type of particle. Enzymes that were adsorbed on the particles maintained their activity (Table 2).

Coccolith particles are composed of inorganic calcite ($CaCO_3$) and a small amount of organic matter (*9,12*). The small size of complex-shaped calcite particles and the presence of the small amount of organic materials are properties that make these calcite particles suitable for a wide range of technically and medically important applications, a few of which are starting to be recognized. These calcite particles have high surface areas and easily disperse in solution. Therefore, these calcite particles may be conjugated to biologically active substances. In addition to enzyme immobilization, immunoassay for the determination of immunoglobulin G using FITC conjugated antimouse IgG antibody immobilized on these calcite particles has been performed (data not shown). Other applications, including the use of coccolith particles in semiconductors, fine ceramics, and medicines, are currently under investigation. Coccolith ultrafine particles are also being isolated from coccolithophorid algae other than *E. huxleyi* and *P. carterae*. Algal calcite ultrafine particles should be treated as new and valuable biological resources for high-technology

application. Future work is also being directed toward large-scale production of coccolithophorid algal biomass using photobioreactors that employ light-diffusing optical fiber (*13,14*).

REFERENCES

1. Outka, D. E. and Williams, D. C. (1971), *J. Protozool.* **18,** 285–297.
2. van der Wal, P., de Jong, E. W., and Westbroek, P. (1983), *Plotoplasma* **118,** 157–168.
3. van der Wal, P., de Jong, E. W., Westbroek, P., and de Bruijin, W. C. (1983), *Environ. Biogeochemistry* **35,** 251–258.
4. van der Wal, P., de Jong, E. W., Westbroek, P., de Bruijin, W. C., and Mulder-Stapel, A. A. (1983), *J. Ultrastruct. Res.* **85,** 139–158.
5. Paasche, E. (1962), *Nature* **193,** 1094, 1095.
6. Paasche, E. and Klaveness, D. (1970), *Arch. Microbiol.* **73,** 143–152.
7. Nielsen, E. S. (1966), *Physiologia Plantarum* **19,** 232–240.
8. Sikes, C. S., Roer, R.D., and Wilbur, K. M. (1980), *Limnology Oceanography* **25(2),** 248–261.
9. de Jong, E. W., Bosch, L., and Westbroek, P. (1976), *Eur. J. Biochem.* **70,** 611–621.
10. Eppley, R. W., Holmes, R. W., and Strickland, J. D. H. (1967), *J. Exp. Marine Biol. and Ecol.* **1,** 191–208.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265, 266.
12. de Jong, E., van Rens, L., Westbroek, P., and Bosch, L. (1979), *Eur. J. Biochem.* **90,** 560–567.
13. Matsunaga, T., Takeyama, H., Sudo, H., Oyama, N., Ariura, S., Takano, H., Hirano, M., Burgess, J. G., Sode, K., and Nakamura, N. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 157–167.
14. Takano, H., Takeyama, H., Nakamura, N., Sode, K., Burgess, J. G., Manabe, E., Hirano, M., and Matsunaga, T. (1992), *Appl. Biochem. and Biotechnol.* **34/35,** 449–458.