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INHIBITION OF CRYSTAL GROWTH IN COCCOLITH FORMATION OF *PLEUROCHRYSIS CARTERAE* BY A POTENT SCALE INHIBITOR, (1-HYDROXYETHYLIDENE) BISPHOSPHONIC ACID (HEBP)

MASASHI ASAHINA[(1)] & MEGUMI OKAZAKI[(2)]

Keywords: Calcification, coccolith formation, (1-hydroxyethylidene) bisphosphonate (HEBP), *Pleurochrysis carterae*, scale inhibitor

ABSTRACT

The unicellular marine alga *Pleurochrysis carterae* (Haptophyta) produces elaborate calcified scales called coccoliths. The elliptical coccoliths are formed in a special apparatus called coccolith vesicles, originating from the Golgi body, under strict cellular control. In this study we investigated the effect of a potent inhibitor of $CaCO_3$ deposition, (1-hydroxyethylidene) bisphosphonic acid (HEBP), on coccolith formation by electron microscopy. This chemical substance inhibited $CaCO_3$ formation *in vitro* at 0.5 μM. Cells were covered with immature coccoliths or almost naked when they were cultured with 0.5 mM or 1 mM HEBP for 7 days. Transmission electron microscopy revealed that $CaCO_3$ nucleation and crystal growth were inhibited by HEBP at the rim of organic base-plate in coccolith vesicles. A partial loss of anvil-shaped elements and/or immature elements on isolated coccoliths were often observed with scanning electron microscope. However, cell growth was scarcely inhibited by 1mM HEBP during 7 days culture experiment. The amounts of two Ca^{2+}-binding acid polysaccharides and their composition were not significantly different between the normal and HEBP-treated cells. These results suggested that HEBP inhibited specifically $CaCO_3$ nucleation and growth in coccolith formation but not the photosynthesis in this strain. HEBP may be an useful tool to elucidate the role of $CaCO_3$ deposition in uptake of inorganic carbon by photosynthesis in calcareous algae.

(1) Department of Biology, Tokyo Gakugei University, Koganei, Tokyo 184-8501, Japan.
Present address: Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan.
(2) Department of Science Education, Tokyo Gakugei University, Koganei, Tokyo 184-8501, Japan,
E-mail:okazaki@u-gakugei.ac.jp
Tel; +81-423-29-7540
Fax; +81-423-29-7540

INTRODUCTION

The unicellular marine algae coccolithophorids (Haptophyta) produce elaborate calcified scales called coccoliths ,which cover their cell surface. The elliptical coccoliths are formed by the deposition of $CaCO_3$ crystals (calcite) on organic base-plates in coccolith vesicles, special apparatus originating from the Golgi body, and are then extruded onto the cell surface (Wilbur & Watabe, 1963; Outka & Williams, 1971). The calcite elements composing a coccolith are different in size and shape, but each crystal grows from a simple rhombic calcite crystal. A V/R model for

crystal nucleation and growth has been proposed in *Emiliania huxleyi* (Young *et al.*, 1992) and in *Pleurochrysis carterae* (Okazaki *et al.*, 1998). It was suggested that the Ca^{2+}-binding acid polysaccharides associated with coccoliths play an important role in $CaCO_3$ nucleation and crystal growth (de Jong *et al.*, 1976; Westbroek *et al.*, 1984; Young *et al.*, 1992; Marsh, *et al.*, 1992; Okazaki *et al.*, 1998). However, no direct experimental evidence has been reported.

In this study we investigated the effect of a potent inhibitor for nucleation and crystallization of $CaCO_3$, (1-hydroxyethylidene) bisphosphonic acid (HEBP) (Nancollas & Sawada, 1982) on coccolith formation of *Pleurhocrysis cartere* using both scanning and transmission electron microscopes.

MATERIALS AND METHODS

1. Effects of HEBP on $CaCO_3$ precipitation *in vitro*

The rate of $CaCO_3$ formation was determined by recording the decrease in pH in the reaction medium as described previously (Okazaki *et al.*,1998), according to the method of Wheeler *et al.* (1981). Three milliliters of 20 mM $CaCl_2$ was added to 3 ml of 20 mM $NaHCO_3$ (pH 8.8) and either 0.3 ml of H_2O (control) or 0.3 ml of a solution of (1-hydroxyethylidene) bisphosphonic acid (HEBP) (Wako Co., Japan) (Fig. 1) at a given concentration. Alternatively, the HEBP solution was added 3 min after onset of precipitation. The pH was measured at 30-s intervals over 30min by a combined pH glsass electrode (GST-155C, Toa Denpa Kogyo Co.) and a pH meter (HM-5A, Toa Denpa Kogyo Co.).

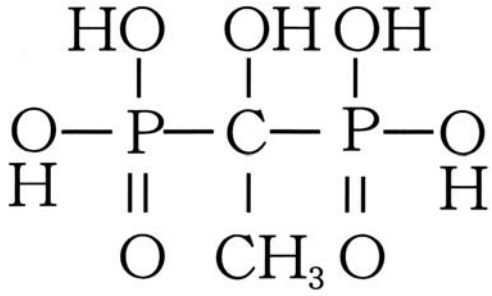

Figure 1.
Molecular structure of (1-Hydroxyethylidene) bisphosphonic acid (HEBP). Molecular weight =206.

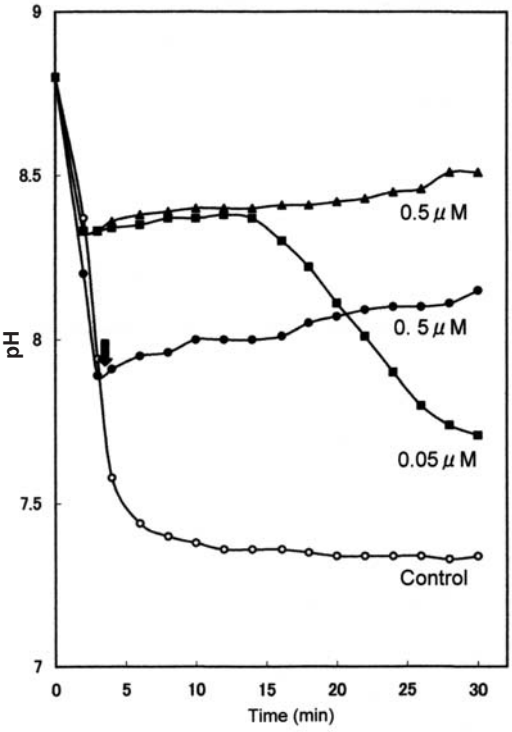

Figure 2.
Effects of HEBP on $CaCO_3$ precipitation in vitro.
Three milliliters of 20 mM $CaCl_2$ was added to 3 ml of 20 mM $NaHCO_3$ (pH8.7) and 0.3 ml of H_2O (control) or 0.3 ml of a solution of HEBP at a given concentration (0.05 μM, 0.5 μM). Alternatively, the HEBP solution was added after 3 min after onset of precipitation (arrow).

2. Plant materials and growth conditions

An axenic clone of *Pleurochrysis carterae* was kindly provided by Dr. E.W. de Vrind- de Jong (Department of Biochemistry, Leiden University, The Netherlands). Cells were grown in 100-ml or 5, 000-ml flasks containing seawater-based medium, as described by Eppley *et al.* (1967), with or without HEBP (0.5 mM or 1 mM) for 12 days. The cultures were grown under an 18-hr light /6-hr dark cycle (19°C) at an illumination of approximately 100 μmol m^{-2} s^{-1}. The 100 ml culture in a 300 ml-flask was stirred gently every day, but 4,000 ml culture in a 5,000 ml-flask was aerated continuously. Cell numbers were counted at 2 or 3days intervals using a hemocytometer by taking out 1ml of the culture media in a 300ml-flask.

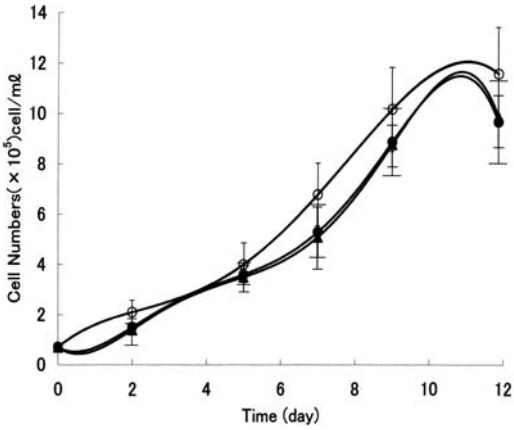

Figure 3.
Effect of HEBP on algal growth.
The culture were grown in 100 ml of the medium containing 0.5 mM or 1mM HEBP under a light intensity of approximately 100 μmol $m^{-2}s^{-1}$ (18 hr-light/6-hr dark) with gentle starring every day. Cell numbers were counted using a hemocytometer and expressed as cell numbers/ml of the cultute.
Open circle: without HEBP (control), closed circle: 0.5 mM HEBP, closed triangle: 1mM HEBP.

3. Electron microscopy

For the transmission electron microscopy (TEM), algal cells cultured for 7 days were collected by centrifugation and fixed in 5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr. Samples were washed in the same buffer and post-fixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) at 4°C overnight. After fixation, samples were washed in 0.1 M cacodylate buffer (pH 7.4), dehydrated in a graded ethanol series, and embedded in Epon 812 resin. Ultra-thin sections were prepared using a diamond knife on an ultramicrotome (JEOL, EM Super Nova) and placed on copper grids. Sections on grids were double-stained with uranyl acetate and lead citrate. Observations were made using a transmission electron microscope (JEOL, JEM 100 CX-II) at an accelerating voltage of 80kV.

For the scanning electron microscopy (SEM), cells were collected on an isopore membrane filter (0.2 μm) (Millipore, MA, USA) by suction. After fixation and dehydration as described above, the specimens were dried in a freeze dryer (JEOL, JFD-310), and then coated with gold (Au). Observations were made using a SEM (JEOL, JSM 5800 LV) at an accelerating voltage of 15kV.

Coccoliths were isolated from cells as described previously (Okazaki *et al.*, 1998), coated with Au, and observed with a SEM (JEOL, JSM 6301F), or observed with the TEM as described above, after shadowed with Pt-Pd at an angle of 30°.

4. Preparation of acid polysaccharide from cell and polyacrylamide gel electrophoresis

Acid polysaccharides were prepared by the method of Okazaki *et al.* (1998). Cells in 7 days-cultures (each 4, 000 ml) with 1mM HEBP or without HEBP (control) were collected by centrifugation at

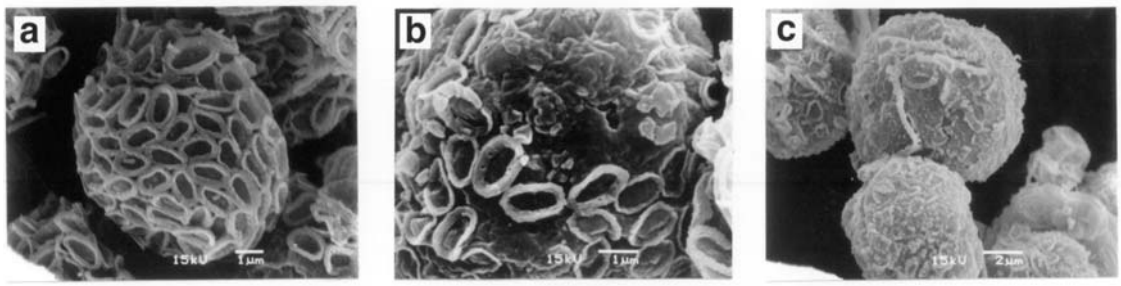

Figure 4. *Scanning electron micrographs of P. carterae.*
a: control cell (cultured without HEBP), b:cell cultured with 0.5mM HEBP, c: cell cultured with 1mM HEBP.

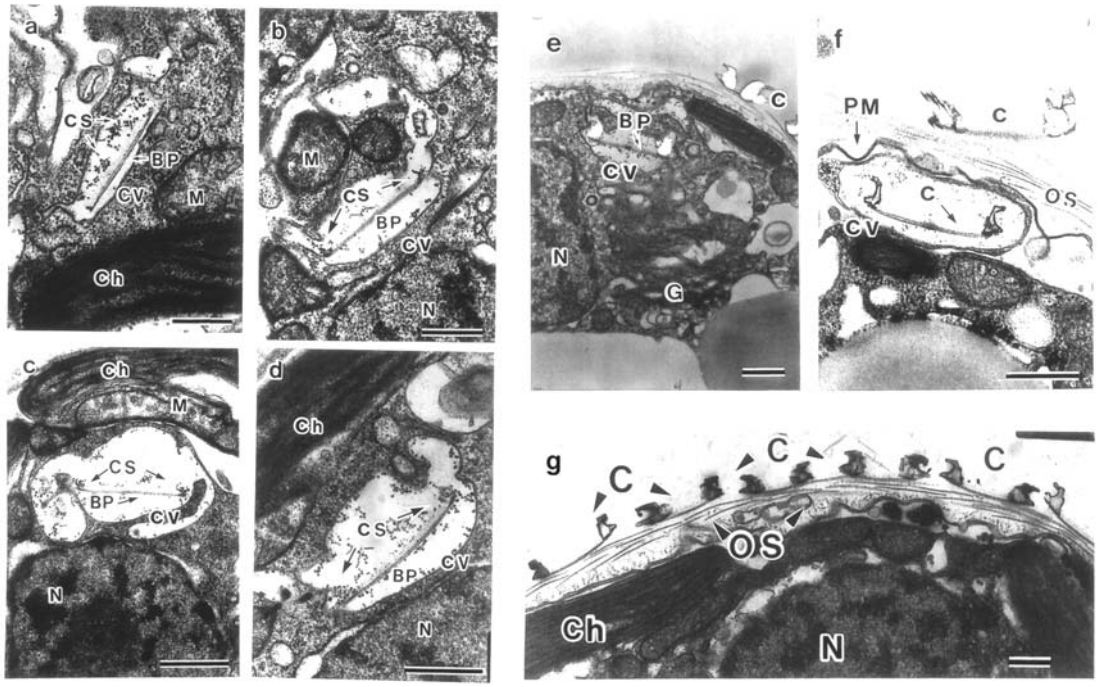

Figure 5.
Transmission electron micrographs showing a sequential coccolithogenesis in the control cells.
BP: base-plate, C: coccolith, Ch: chloroplast, CS: coccolithosomes, CV: coccolith vesicle, G: Golgi body, M: mitochondria, N: nucleus, OS:organicscale, PM: plasma membrane. Stained with U - Pb. Scale bars = 0.5 μm.

8,000 xg and decalcified in 10% EDTA (pH 8.0) and insoluble residues were removed by centrifugation at 15,000 xg. The supernatant was dialyzed in cellulose tube against distilled water. The dialyzate was concentrated to dryness by a rotary evaporator at 40°C. The dried material was dissolved in 0.05 M Tris-HCl buffer (pH 7.5). The soluble fraction was applied on a column of DEAE-cellulose equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) and eluted with a 0 to 0.3 M NaCl gradient. The peaks positive to a carbazole-H_2SO_4 were collected and lyophilized after dialysis against distilled water. The polysaccharides containing uronic acid were determined by the carbazole-H_2SO_4 method, and the amount was calculated from a curve calibrated with glucuronic acid.

Polyacrylamide gel electrophoresis was performed as described previously (Okazaki *et al.*, 1998).

RESULTS

1. Effect of HEBP on $CaCO_3$ formation *in vitro*

HEBP is a kind of phosphonates (M.W. = 206) and a potential scale inhibitor of calcite and aragonite (Fig. 1) (Nancllas & Sawada, 1982). This inhibitor showed a strong inhibitory effect on $CaCO_3$ precipitation even at 0.5 μM (Fig.2). It delayed $CaCO_3$ precipitation for at least 30 min at 0.5 μM.

2. Effect of HEBP on algal growth

Figure 3 shows the growth curves when cultured with 0.5 mM or 1mM HEBM and without HEBP (control). No significant difference in cell numbers was observed between control and HEBP-treated cells at 12 days. The cell size was not affected by this inhibitor (Fig. 4a,b,c).

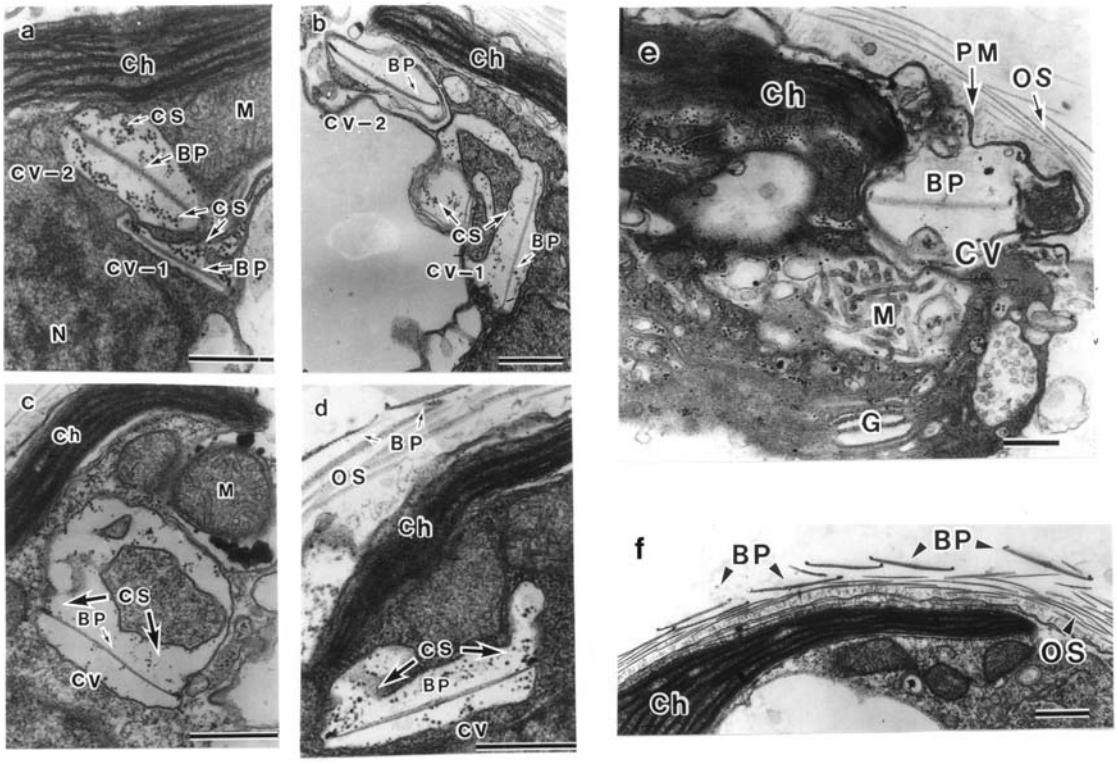

Figure 6.
Transmission electron micrographs showing a sequential coccolithogenesis in the cells cultured with 1mM HEBP. BP: base-plate, C: coccolith, Ch: chloroplast, CS: coccolithosomes, CV: coccolith vesicle, G: Golgi body, M: mitochondria, N: nucleus, OS:organic-scale, PM: plasma membrane. Arrows shows ridges of BP. Stained with U - Pb. Scale bars = 0.5 µm.

3. Electron microscopy

The effect of HEBP on coccolith formation in *P. carterae* was obseved by electron microscopy. SEM observations revealed that HEBP inhibited $CaCO_3$ deposition on the organic base-plates. Most cells lost their coccoliths partially when cultured with 0.5mM HEBP for 7 days and remarkably with 1mM HEBP (Fig. 4b, c). The latter cells were covered with very poorly calcified scales (Fig. 4c).

TEM observations were carried out to examine the effects of HEBP on intracellular coccolith development. In cells cultured without HEBP, normal coccolith development occurred as described by Outka & Williams (1971) (Fig. 5a-g). Sequential stages of coccolithogenesis were observed as followed; pre-stage (Fig. 5a), beginning stage (Fig. 5b), early stage (Fig. 5c), middle stage (Fig. 5d), late stage (Fig. 5e), and final stage (Fig. 5f). $CaCO_3$ nucleation and crystal growth occurred at the rim of organic base-plate (BP) in the coccolith vesicles (CV), and the shape of the vesicles changed during coccolithogenesis. Prior to mineralization it was flat and its volume was smaller than at later stages (Figs. 5a). Many electron-dense granules identified as coccolithosomes (Outka & Williams,1971) were apparent from the pre-stage to middle stage. During mineralization the vesicle was swollen and had peripheral extensions (Figs. 5b-d). After mineralization it was more swollen, and the peripheral extensions as well as coccolithosomes disappeared (Fig. 5f). Finally the mature coccoliths were extruded onto the cell surface (Fig. 5g).

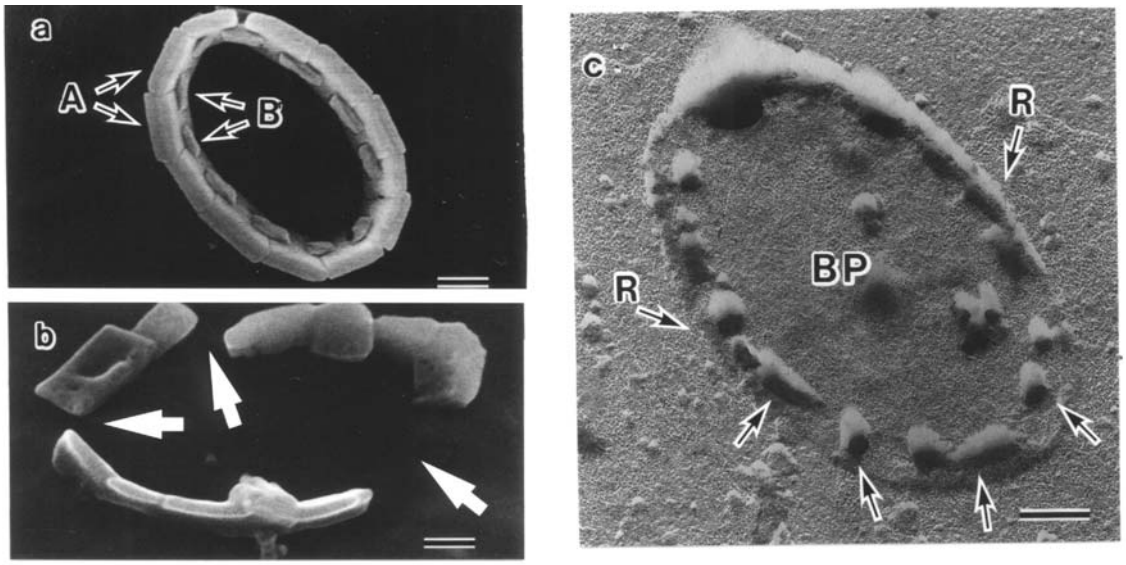

Figure 7.
Scanning and transmission electron micrographs of isolated coccoliths.
a: coccolith from control cell, b and c: coccolith from cell cultured with 1mM HEBP. c: specimen shadowed with Pt-Pd at an angle of 30°.
R: rim of base-plate, A: large element, B: small element. White and black arrows show lack of elemental crystals and early developmental stage of crystals, respectively. Scale bars = 0.1 μm.

In the cells cultured with 1mM HEBP, several stages of coccolithogenesis were also identified; pre-stage (Fig. 6a;CV-1), initial stage (Fig. 6b; CV-1), early stage (Fig. 6c), middle stage (Fig. 6d), late stage (Fig. 6a; CV-2) and final stage (Fig. 6b; CV-2, Fig. 6e). Coccolithosomes and Golgi body appeared normal. However, in sharp contrast to control cells (Fig.5a-g), HEBP inhibited $CaCO_3$ deposition at the rims of the base-plates in the coccolith vesicles (Fig. 6b; CV-2, Fig. 6e). As a consequence, non-calcified base-plates were seen on top of the thin organic scales on the cell surface (Fig.6f).

SEM (Figs. 7a, 7b) and TEM (Fig. 7c) observations of isolated coccolith were carried out. The coccolith consists of about 12 anvil-shaped large (A) and small (B) elements situated on the rim of organic base-plate; and the elements alternate and interlock (Outka & Williams, 1971; Okazaki *et al.*, 1998). Fig. 7a shows an intact coccolith isolated from control cells. In contrast, coccoliths prepared from cells cultured with 1mM HEBP showed an abnormally calcified base-plate with immature anvil-shaped crystals, and some elements were lacking from the plate (Fig. 7b, arrows). TEM observation of Pt-Pd shadowed specimens revealed an organic base-plate with early developmental stage of crystals due to an inhibition of crystal growth with HEBP (Fig. 7c, arrows).

4. Effects on the biosynthesis of Ca^{2+}-binding acid polysaccharides

We investigated the effect of HEBP on the biosynthesis of Ca^{2+}-binding acid polysaccharides (Marsh *et al.*, 1992; Okazaki *et al.*, 1998). The polysaccharides were extracted with EDTA from normal cells and from those cultured with 1mM HEBP for 7 days and were isolated by DEAE-cellulose column chromatography. Fig. 8 shows an elution profile of acid polysaccharides from a DEAE-cellulose column. Both normal and HEBP treated cells contained similar amounts of two acid polysaccharides (A and B) as reported previously by Okazaki *et al.* (1998). Their amounts in whole cells showed no significant difference between normal and HEBP-treated cells. In addition, acid polysaccharides A and B from both cells showed the same profile on polyacrylamide gel eletrophoresis (PAGE) (Fig. 9).

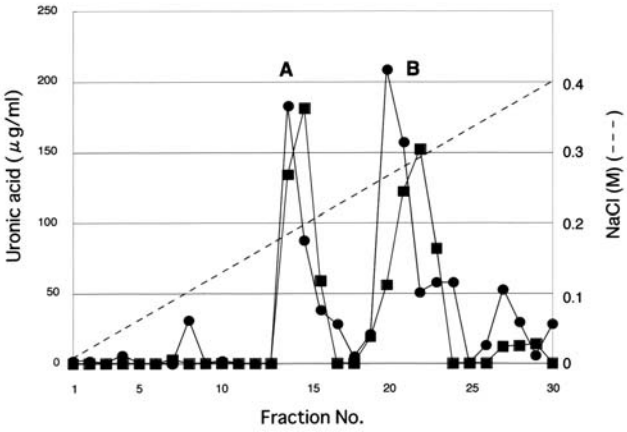

Figure 8.
DEAE-cellulose column chromatography of EDTA-soluble acid polysaccharides from control cells and cells cultured with 1mM HEBP. Closed circle: acid polysaccharides from control cells, closed square: acid polysaccharides from cells cultured with 1mM HEBP, A and B: two components of acid polysaccharides.

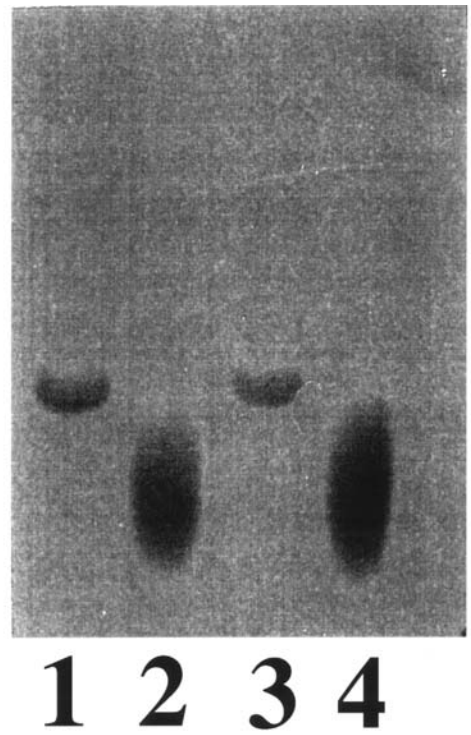

Figure 9.
Polyacrylamide gel electrophoresis of isolated acid polysaccharides from control cells and ones cultured with 1mM HEBP. Lane 1and 2: acid polysaccharide A and B from control cells, respectively. Lane 3 and 4: acid polysaccharide A and B from cells cultured with 1mM HEBP, respectively. Approximately 50 μg of acid polysaccharide was applied to each lane. Gel was stained with alcian blue in 0.75% acetic acid.

DISCUSSION

In this study, we showed that coccolith formation of *P. carterae* was remarkably inhibited by HEBP added to culture medium at 1mM. This inhibition may have been caused due to the inhibition of biosynthesis of Ca^{2+} -binding acid polysaccharides by HEBP, because a Ca^{2+}-binding acid polysaccharide (PS-2)-deficient strain of *P. carterae* did not form coccolith (Marsh & Dickinson , 1997). The PS-2 seemed to correspond to our component B (Okazaki *et al.*, 1998). However, this possibility was excluded because there was no significant difference in contents and composition of the acid polysaccharides between normal cells and cells cultured at 1 mM HEBP (Figs. 8 and 9). Thus, it was concluded that HEBP penetrating into coccolith vesicles inhibited nucleation and /or crystal growth of $CaCO_3$ at the rim of organic base-plate in coccolith vesicles. To our knowledge, this is the first report that HEBP can be used as an inhibitor for coccolith formation.

Many studies have discussed some relationship between $CaCO_3$ deposition in coccolith formation and photosynthesis (Paashe,1964; Nimer & Merrett, 1992) in coccolithophrids and in macro calcareous marine algae (Borowitzka,1982). Our present study showed that HEBP hardly inhibited the cell growth of *P. carterae* (Fig. 3), suggesting not only no significant inhibition of photosynthesis, but also a lack of coupling of calcification with uptake of inorganic carbon by photosynthesis. We are now studying the effects of HEBP on photosynthesis of calcareous and non-calcareous algae to elucidate the role of $CaCO_3$ deposition for uptake of inorganic carbon by photosynthesis.

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