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EFFLUENTS FROM INTENSIVE CULTURE PONDS OF TIGER PRAWN (*PENAEUS MONODON* FABRICIUS)

Yont Musig, Wanna Ruttanagosrigit and
Suksri Sampawapol

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CHEMICAL COMPONENTS OF RED-BROWN MATERIAL IN CYST OF *SCRIPPSIELLA TROCHOIDEA* (DINOPHYCEAE)

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ABSTRACT

Marine dinoflagellate *Scrippsiella trochoidea* (Stein) Loeblich III was induced to form cyst under laboratory conditions. The cysts of dinoflagellate generally have red-brown material containing in cytoplasm. This present study attempted to examine the chemical components of this material. The results demonstrated that the red-brown material composed of fucoxanthin, 19'-hexanoyl fucoxanthin, diadinoxanthin and diatoxanthin. Furthermore, glutamic acid and glycine were found to be the dominant amino acid components of the red-brown material. The result was also revealed that the red-brown material was not composed of polysaccharides. The results from the observation of red-brown material after cyst germination suggesfed that the red-brown material may be useful for growth of vegetative cell after germination from cyst.

INTRODUCTION

In general, dinoflagellates produce cyst through the sexual phase in their life histories. The widely reported inclusion of lipid globules or starch grain in cysts suggests that the production of storage compounds accompanies cyst formation. One of the special characteristics of phytoflagellate cysts is a red-brown spot containing in cytoplasm (Coats et al., 1984; Anderson et al., 1988; Fritz et al., 1989; Gao et al., 1989; Ellegaard et al., 1993). It may be called as "red body", "dark-brown material" etc. In this study, it was called as " red-brown material". The red-brown material was formed after the formation of planozygotes had been formed. Although cyst had already germinated, this material still remained in the newly germinated cell. This material disappeared in a few days after the germination. The possibility that red-brown material containing with pigments has been suggested by a number

of authors (Turpin et al., 1978; Coats et al., 1984; Ellegaard et al., 1993). However, the chemical components and the role of red-brown material in cyst remain largely unknown, partly because of the difficulty in separating this material from the whole cyst.

In this study, the components of amino acid pool, pigment and polysaccharide of red-brown material in cyst of *Scrippsiella trochoidea* were determined. The observation of the changes in red-brown material after cyst germination by using invert light microscope was also performed.

MATERIALS AND METHODS

Experimental Design

Clonal axenic culture strain of *Srcippsiella trochoidea* (STEIN) LOEBLICH were precultured for two weeks in 300 ml Erlenmeyer flasks containing 150 ml of autoclaved (120 $^{\circ}$C, 20 min) ESM-enriched seawater from the Seto Inland Sea (salinity 31±1 ‰) without soil extract under culture conditions (Meksumpun et al., 1994a). One hundred ml of stock culture was inoculated into 2 l Erlenmeyer flask containing 1 liter of nitrogen and phosphorus depleted ESM-medium (Meksumpun et al., 1994b). Cysts of *S. trochoidea* were harvested about three weeks after the inoculation. They were separated from vegetative cells with a Percoll-sorbitol solution step gradient centrifugation. Cysts were treated with 16 N sulfuric acid for 30 min on ice and washed several times with distilled water by centrifugation before chemical analyses. The red-brown material was remained in cyst after treated with sulfuric acid.

Pigment Analysis

Cysts were carefully transferred to microhomoginizer tube and ground with glass pestle by hand for 5 min. After grinding, cysts were extracted the pigment in 2 ml dimethylformamide (DMF). The pigment extracts were analyzed for pigments by using HPLC system (Jusco 880-PU intelligent HPLC pumps, Jusco 870-UV intelligent UV/VIS detector, using a wavelength of 440 nm). The column was Tosoh TSK-GEL ODS 80Tm analytical column (250 x 4.6 mm ID). As eluents, buffers A and B were used: buffer A was 70%

methanol containing 25.5 mM tetrabutyl ammonium acetate ; buffer B was ethylacetate containing 80% methanol (v/v). The elution profile was the following: a 10 min concave gradient from 50% buffer A to 100% buffer B; 30 min isocratic elution with 100% buffer B. The flow rate was 0.8 $ml \cdot min^{-1}$. The column temperature was 35 $^{\circ}C$.

Amino Acid Analysis

After grinding, cysts were hydrolyzed with 6 N hydrochloric acid at 105 $^{\circ}C$ for 22 hours. The hydrolysate was evaporated to dryness under vacuum, redissolved in distilled water. Samples were analyzed for amino acids using reversed phase high performance liquid chromatography (HPLC). The HPLC system used in this study following the system of Meksumpun et al. (1993).

Polysaccharide Analysis

Cysts were homogenized in distilled water using glass microhomogenizer and then centrifuged (2000 x g) for 10 min. The pellet was removed lipid and pigment by resuspending in chloroform-methanol (2:1, v/v). Methanol-fraction containing with polysaccharides was combined with the first supernatant. The pellet was again extracted with hot 80% ethanol and then centrifuged, supernatant was discarded and residue was extracted three times with hot distilled water. The hot water soluble polysaccharides were combined with first supernatant and methanol fractions. The dissolved polysaccharides were precipitated with 4 volumes of ethanol. They were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 $^{\circ}C$ for 12 hours after dialysis and freeze dried. Subsequent reduction, acetylation, and analysis of alditol acetates of sugar by gas-liquid chromatography (Hitachi 263-30; column : 3% SP-2340/ Uniport Hp (3 mm x 2 m); column temp : 190-250 $^{\circ}C$ (4 $^{\circ}C \cdot min^{-1}$) following the method of Okutani (1984).

Observation of Red-brown Material after Cyst Germination

Sediment was collected from Hiketa Bay (Eastern part of the Seto Inland Sea) by

gravity core sampler. The sediment core samples were cut and removed for the upper 0-2 cm. The sediment was resuspended in autoclaved seawater and filled into the 100 ml Erlenmeyer flask. Subsamplings were done every 3 hours interval for two days by removing the overlying seawater in the flask and observed newly germinated cells under invert light microscope. The newly germinated cells were directly picked up and transferred to a 1 ml microtubes containing with 0.5 ml of autoclaved seawater by micropipette. The microtubes were separated to 2 sets and incubated under culture conditions or in darkness. The red-brown material in newly germinated cell was observed every days for three days.

RESULTS

Red-brown material in *Scrippsiella trochoidea* cyst is shown in Fig. 1A. It was found that after cysts were treated with sulfuric acid at the concentration between 12 and 16 N for about 30 min in ice, spines and some parts of cyst wall were dissolved, whereas the red-brown material still remained in cyst (Fig. 1B, C). The concentration of 16 N H_2SO_4 is likely to be the optimum concentration to obtain the red-brown material. The red-brown material was completely extracted by DMF (Fig. 1D). Figure 2 showed the chromatograms of pigments in whole cyst and in red-brown material. The red-brown material composed of fucoxanthin, 19'-hexanoyl fucoxanthin, diadinoxanthin and diatoxanthin. Interestingly, chlorophyll *a*, chlorophyll *b*, chlorophyll *c* and b-carotene cannot be detected in the red-brown material. Compositions of pigments in the red-brown material compared to the whole cyst were shown in Fig. 3. Let the contents of each pigment in whole cyst be 100%, diatoxanthin and 19'-hexanoyl fucoxanthin contents in the red-brown material were higher than 60%. The contents of fucoxanthin and diadinoxanthin were higher than 30 and 40%, respectively.

Figure 4 showed the chromatogram of standard amino acids and amino acids in the red-brown material. The composition of amino acids in the red-brown material was shown in Fig. 5. Glutamic acid and glycine were found to be the dominant amino acid components of the red-brown material (about 15%). The relative abundances of other amino acids varied among 3-11% except tyrosine which was only 1%. Let the composition of each amino acid in

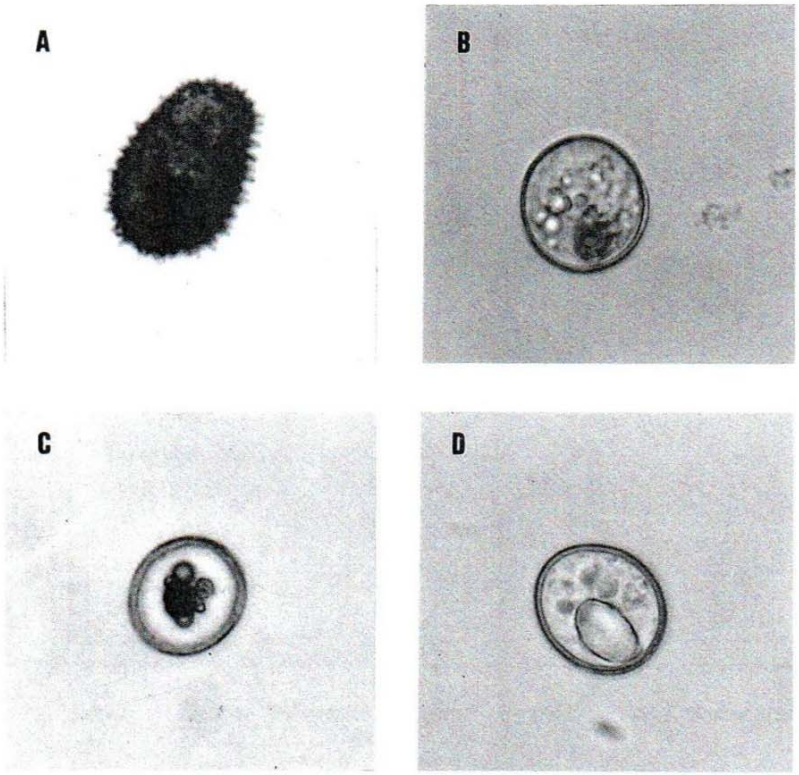

Figure 1 Red-brown material of *Scrippsiella trochoidea* cysts; Red-brown material in normal *S. trochoidea* cysts (A), after treatment with 12 N H_2SO_4 (B), after treatment with 16 N H_2SO_4 (C), after extraction with dimethylformamide (D).

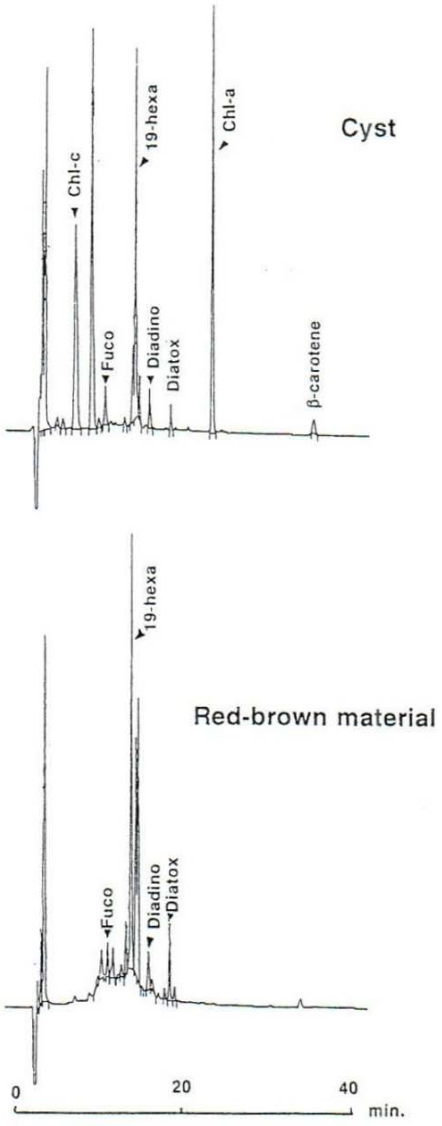

Figure 2 Reverse phase HPLC chromatograms of pigments in cyst (upper) and red-brown material (lower).

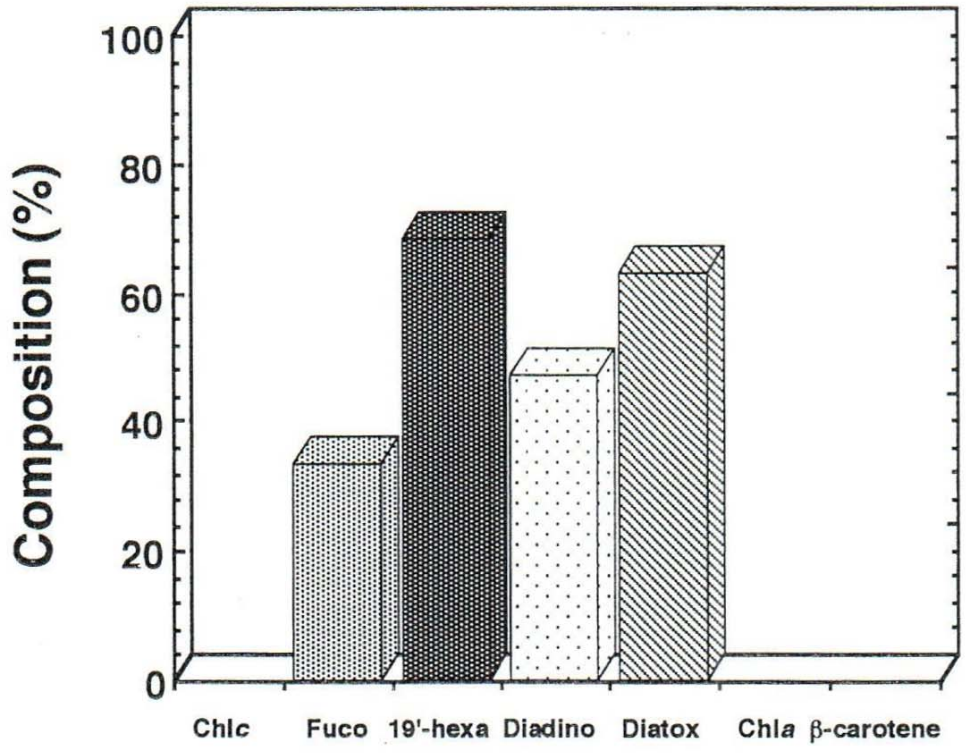

Figure 3 Pigment composition in red-brown material (let the content of each pigment in whole cyst be 100%).

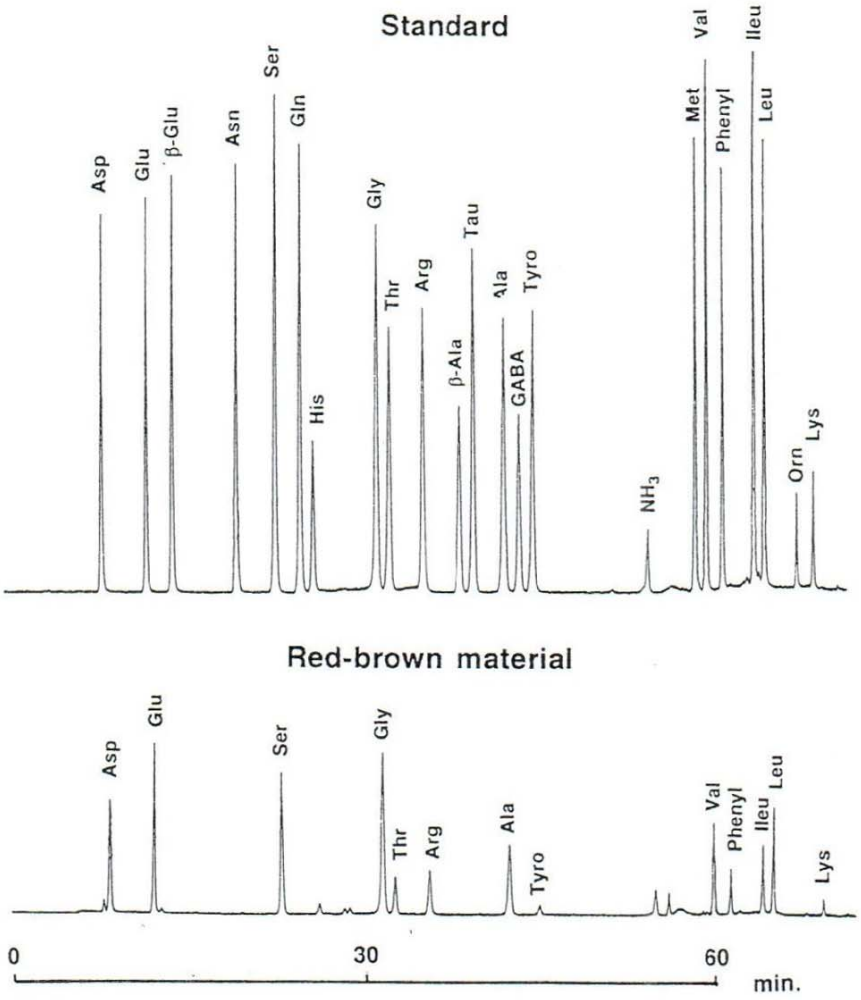

Figure 4 Chromatograms of standard amino acids and amino acid components in red-brown material.

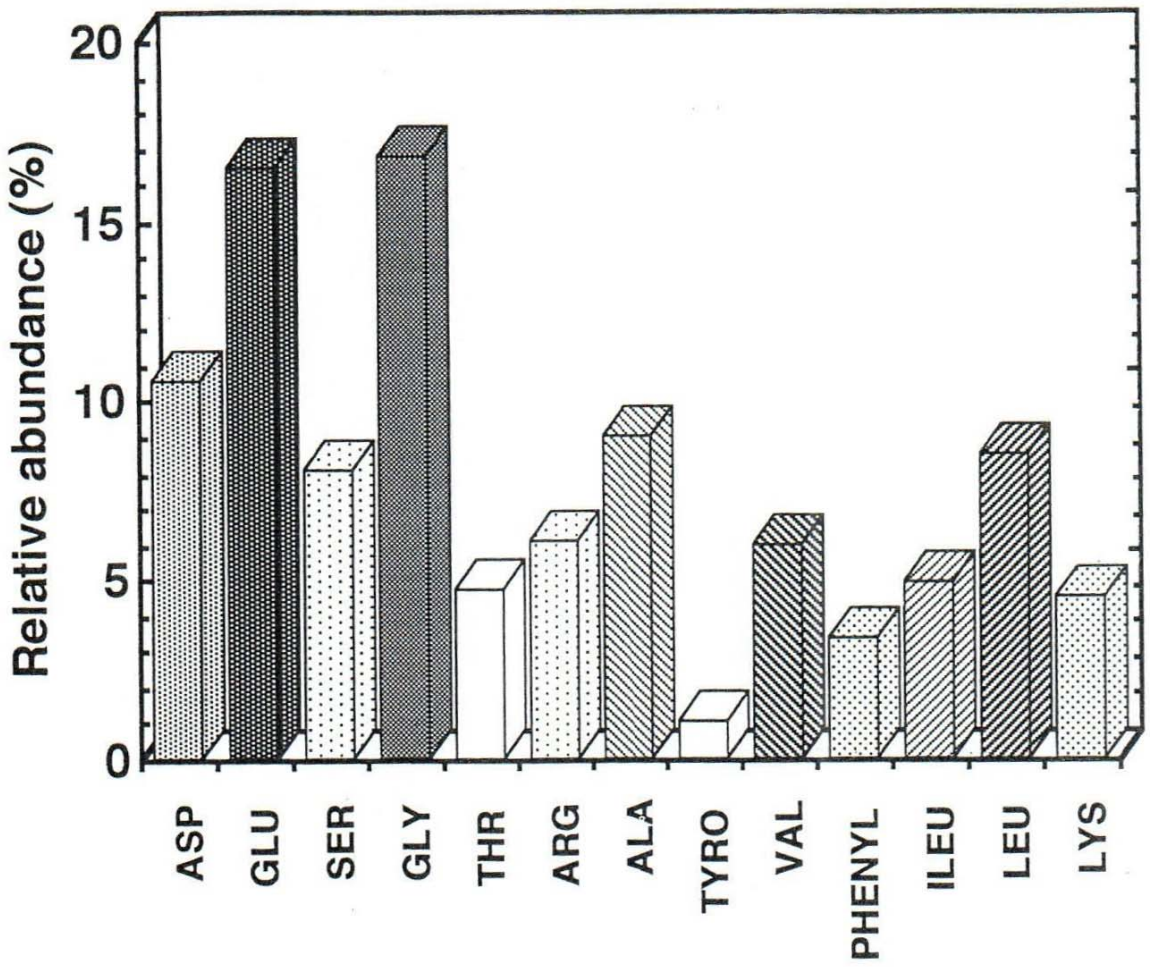

Figure 5 The amino acid composition of red-brown material.

whole cyst be 100%, the percentages of almost amino acids were about 30% except lysine which was higher than 70% (Fig. 6).

Figure 7 showed the chromatogram of standard monosaccharides and monosaccharides in the red-brown material. Although glucose was usually found in phytoflagellate cells, in this study, glucose cannot be detected in the red-brown material at all.

The newly germinated cells after incubation under culture conditions and in darkness were shown in Fig. 8. Under dark condition, the red-brown material still remained in cell after germination for three days. However, it disappeared after germination for two days under the culture conditions.

DISCUSSION

In this present study, an attempt to determine the chemical components of red-brown material in *Scrippsiella trochoidea* using H_2SO_4 for separating the red-brown material from the cytoplasm indicates that the optimal concentrations of H_2SO_4 are between 14 and 16 N. Although some chemical components may still remain in cyst. The sample should be good representative of the red-brown material because the observation of this material under light microscope demonstrated that cyst was bursted after acid treatment and almost of cytoplasm was dissolved in acid except the red-brown material.

The results clearly showed that the red-brown material was mainly composed of carotene and xanthophyll only. It has been reported that many marine nonphotosynthetic dinoflagellates belonging to the genus *Protoperidinium* contained red-colored droplets in the cytoplasm, which are probably carotenoid in nature. However, there has no report about the pigment composition of the red-brown material. This material just has been proposed as the pigment accumulation by many researchers (Coats et al., 1984; Anderson et al., 1988; Ellegaard et al., 1993). Gao et al. (1989) observed the red-brown material (red body) using Transparency Electron Microscope (TEM) and proposed that the red-brown material in *Scrippsiella* sp. may result from development of the membrane-bound body. This membrane-bound is similar to the accumulation body of *Woloszynskia tylota* cysts, contains dense

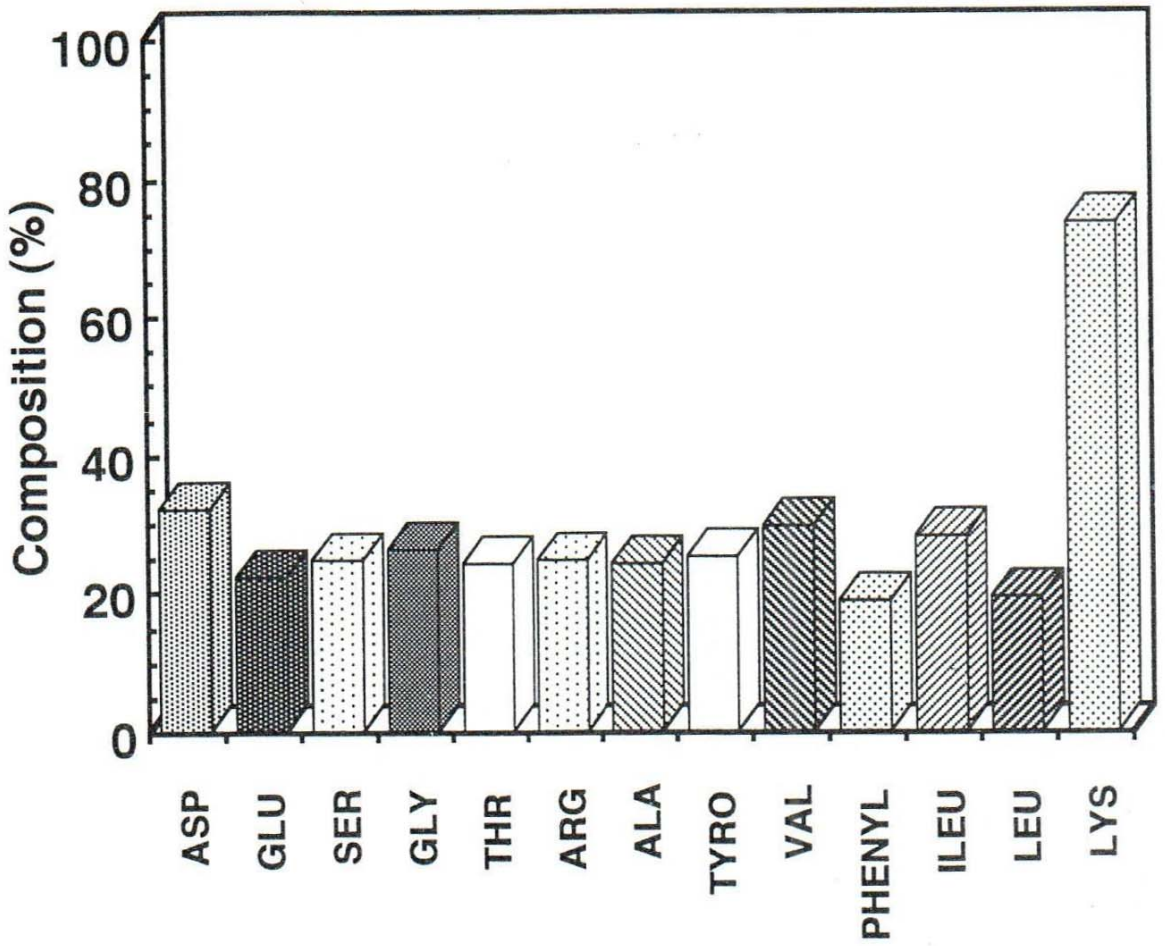

Figure 6 The amino acid composition of red-brown material (let the composition of each amino acid in whole cyst be 100%).

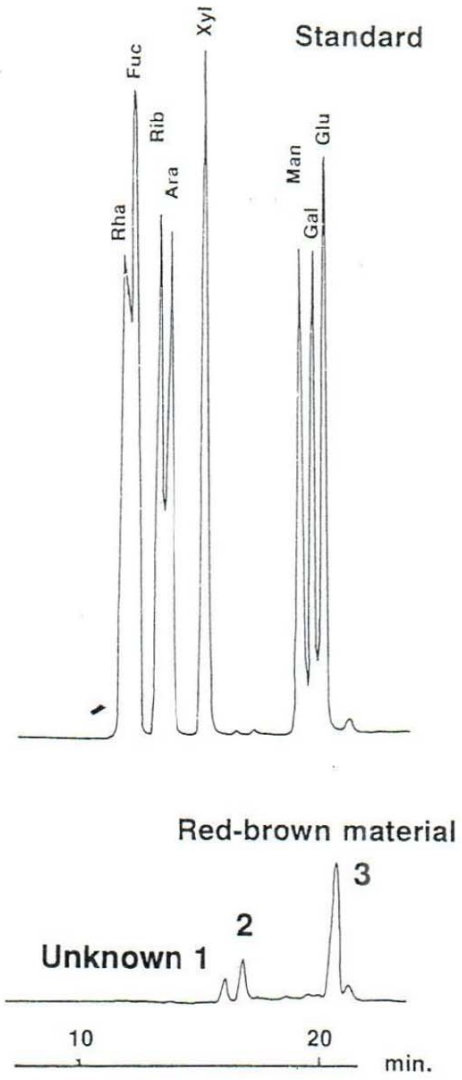

Figure 7 GLC separation of standard sugar and the hydrolyzed cold and hot water extracted from the red-brown material.

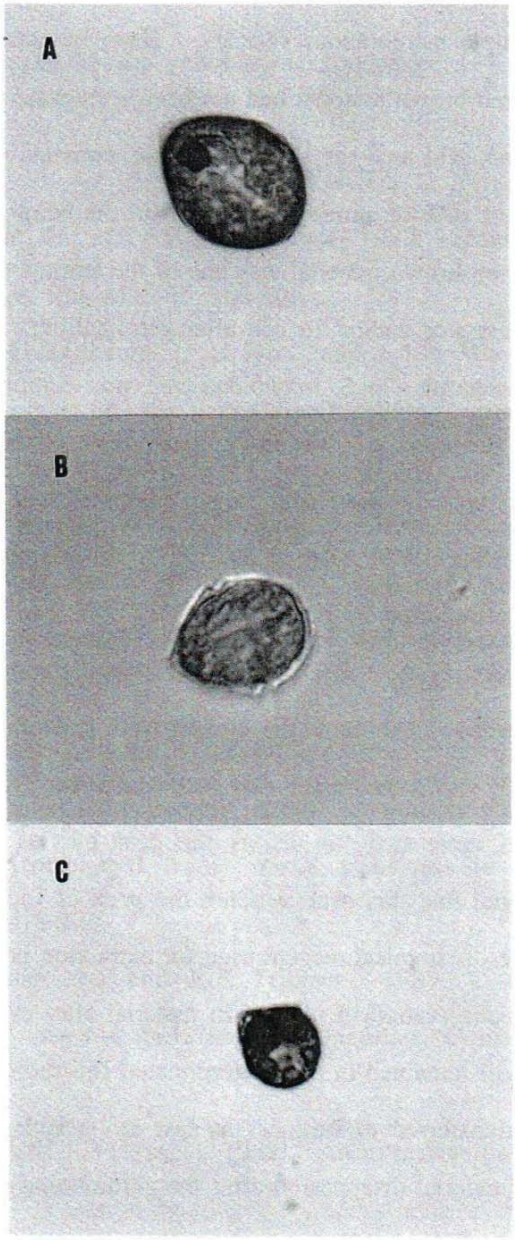

Figure 8 Newly germinated cells of *S. trochoidea*; after germination less than 3 hours (A), after germination for two days under culture conditions (B), after germination for three days under dark condition (C).

amorphous material and multiple membranous vesicles. However, the determination for the chemical composition of the red-brown material had not been conducted in detail.

The result from amino acid analysis showed that the contents of each amino acid of the red-brown material is about 30% of amino acid content of the whole cyst except lycine. It should be concluded that the red-brown material was one of the storage compounds which may be useful for cyst during quiescence and/or for cell after germination. Lirdwitayaprasit et al. (1990) suggested that polysaccharides in *S. trochoidea* cyst was composed of fucose, ribose, xylose, mannose, galactose and glucose. Moreover, many researchers revealed that glucose which in the form of glucan was the dominant sugar component of marine phytoplankton (Boney, 1966; Handa and Yanagi, 1969; Allan et al., 1972). In this study, however, none of monosaccharides that had been reported by Lirdwitayaprasit (1990) can be detected in the red-brown material of *S. trochoidea* cysts. It clearly shows that the red-brown material is not composed of any polysaccharides. Although the highest peak appearing in chromatogram of the red-brown material (Fig. 3) was detected and this peak (unknown 3) had the same retention time as a peak of fucosamine, more study to identify this peak by Gas Chromatography Mass Spectrometer (GCMS) indicated that this peak was not the peak of fucosamine. This peak might be a peak of derivative of chemical reagent used for extraction processes.

The results from the observation of red-brown material after cyst germination showed that the red-brown material still remained in newly germinated cell more than three days if the newly germinated cell was transferred to darkness as fast as possible after the germination. Surprisingly, the red-brown material disappeared after the germination for two days under the culture conditions. It should be concluded that the red-brown material may be useful for growth of vegetative cell after germination from cyst. However, further study on the role of this material should be done.

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