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The Ultrastructure of the Chlorococcalean Picoplankton Isolated from the Western Channel of the Korea Strait

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대한해협에서 분리한 초미세 녹조플랑크톤의 미세구조

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A picophytoplankton has been isolated from the western channel of the Korea Strait. The cell was isolated by dilution method. It is about 2 μm in diameter and has smooth surface. Organelles of nucleus, chloroplasts, mitochondrion, Golgi body, pyrenoids, vacuoles and lipid bodies are identified. Pigments are composed of chlorophyll *a* and chlorophyll *b*, β-carotene and other xanthophylls. Based on the ultrastructural features and pigment composition, it may belong to chlorococcalean picoplankton.

1994년 5월 대한해협 서수로 표층에서 초미세식물플랑크톤을 분리, 배양하였다. 0.2~2.0 μm 범위에서 여과분리 및 희석법으로 단일종으로 분리하여 f/2 배양액으로 배양하였다. 개체의 외형은 구형이었고 지름은 2 μm 내외였으며, 배양된 개체군은 황록색을 보여주었다. 표면은 매끈하였고 세포벽으로 싸여진 세포에서 핵, 엽록체, 미토콘드리아, 골기체, 액포 등이 확인되었다. 분리된 종은 엽록소 *a*와 *b*를 갖는 색소 구성과 세포막, 틸라코이드막 구성, 피레노이드 등을 포함하는 미세구조 특성으로 볼 때 녹조류에 속하는 클로렐라의 일종으로 보인다.

INTRODUCTION

The recognition of minute (0.2~2.0 μm) phototrophic organisms as an important part of the biomass in both marine and freshwater ecosystems was made by Johnson and Sieburth (1979), Waterbury *et al.*(1979) and Murphy and Haugen(1985). These are called picoplankton (Sieburth *et al.*, 1978). Before the joint discovery of chroococcoid cyanobacterial ubiquity in the oceans by Johnson and Sieburth(1979) and Waterbury *et al.*(1979), there were only incidental reports of small, picoplankton-sized cyanobacteria and chlorophyceans in both marine and freshwater ecosystems. Since 1982, the number of reported algal picoplankton occurrences has risen sharply, and at present with the exception of the Black and Red Seas, they have been discovered in all the major oceans of the world (Stockner, 1988).

The picophytoplankton has been recognized for making a significant contribution to primary productivity in the marine environment (Li *et al.*, 1983; Platt *et al.*, 1983; Goericke and Welschmeyer, 1993; Iriarte and Purdie, 1993) as a principal food source for a wide variety of protozooplankton and as a part of the food web in the oceans. For example, the picophytoplankton is grazed by flagellates, ciliates, rotifers, copepods and metazoans, and thus contribute to the flow of energy to higher trophic levels (Stockner, 1988). Marine picophytoplankton includes *Synechococcus* sp., *Chlorella nana, Nano-*

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chloris spp., *Micromonas pusilla, Hillea marina* and so on (Stockner, 1988).

Distributional information obtained to date, however, refers mainly to oligotrophic open ocean waters and comparatively little is known, as yet, about their abundance and significance in the more eutrophic temperate coastal and estuarine waters except several researches (Iriarte, 1993; Madariage and Joint, 1994). In order to have a full and clearer view of the global impact of marine picophytoplankton, more extensive research in these areas is required.

The aspects related to cyanobacteria among the picophytoplankton have been investigated in many ways. For example, their distribution (Waterbury *et al.*, 1979; Johnson and Sieburth, 1982; Takahashi and Hori, 1984; Murphy and Haugen, 1985; Jochem, 1988), primary productivity (Platt *et al.*, 1983; Sheldon *et al.*, 1992; Li, 1994), biomass (Johnson and Sieburth, 1979; Li *et al.*, 1983; Platt *et al.*, 1983; Murphy and Haugen, 1985), the role on microbial loop (Stockner, 1988) and physiological or ecological studies of cyanobacteria were reported. Other picophytoplanktons such as chlorophytes, however, have been scarcely studied except several researches (Dempsey *et al.*, 1980; Johnson and Seiburth, 1982; Takahashi and Hori, 1984; Murphy and Haugen, 1985; Stockner, 1988; Iriarte and Purdie, 1993).

The investigations of nanoplankton and picoplankton have been also recently carried out through the size-fractionated method in Korea (Shin *et al.*, 1990; Choi, 1991; Shim *et al.*, 1991). Shim *et al.*(1991) have reported the contribution of the primary productivity of picophytoplankton in Korean coastal water and there has been growing recognition to their significance as primary producers in the marine environment.

For the present study, a picophytoplankton was isolated at the western channel of the Korea Strait. The basic biological characteristics of the picophytoplankton such as ultrastructure and pigment composition were investigated.

It is likely that the ultimate identification of autotrophic picoplankton requires ultrastructural examination by transmission electron microscopy (TEM), because of their extremely small size. In fact, a lot of investigation for very small sized plankton have been carried out by TEM (Oschman, 1967; Dempsey, 1980; Johnson and Sieburth, 1982; Andreoli *et al.*, 1989; Corpe and Jensen, 1992). To confirm the division of the species, high performance liquid chromatography (HPLC) analysis was also carried out. It offers significant advantages over thin layer chromatography, including speed, automatic detection and much lower detection limits; 0.5 ng for carotenoids and 1ng for chlorophylls with absorbance detection of 84 pg for chlorophylls using fluorescence detection (Wright and Shearer, 1984).

Up to now, the isolation and culture of picophytoplankton as well as the biomass and primary productivity of them were scarcely studied in Korean ocean waters. It is important to isolate and culture picophytoplankton and to investigate the biological characteristics and the role of them on the ocean environment in Korea. The main subject of the present study are to culture the picophytoplankton isolated from the Korea Strait, to examine the ul-

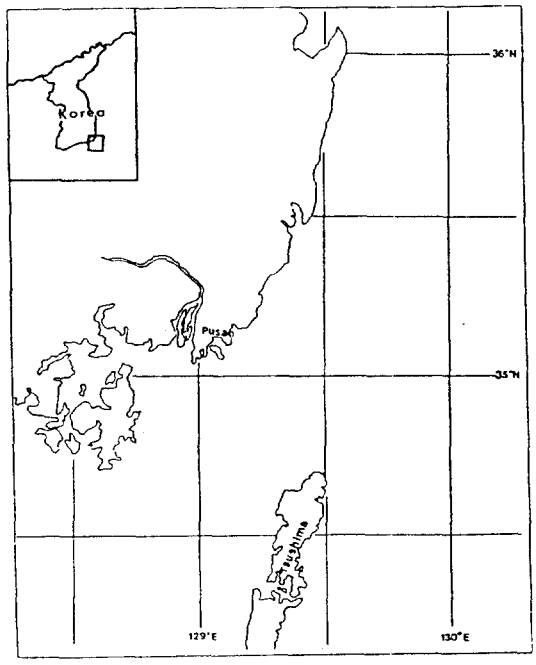

Fig. 1. The sampling site of the present study.

trastucture of the species through electron microscopy and to analyze the pigment composition using HPLC.

MATERIALS AND METHODS

Fieldwork

Sampling was performed in May 12, 1994 in the western channel of the Korea Strait (Fig. 1). Seawater was taken with a submersible pump from 3~5 m below the surface into plastic bottles. For the collection of picophytoplankton, seawater samples were size-fractionated filtered. One liter seawater was serially filtered through 25, 2.5 and 0.2 μm filters. The picophytoplankton fraction isolated on filters was put in enriched seawater, f/2 media (Guillard, 1973). In order to estimate the biomass of picophytoplankton, chlorophyll contents were measured through size-fractionated filtration and subsequent extraction by acetone for the spectrophotometric method (see 4. Pigment analysis).

Isolation and algal culture

The media used in algal culture was f/2 media without silicate (Guillard, 1973). The media added with stock solution to seawater was autoclaved at 121°C for fifteen minutes. The culture was maintained under a continuous light at 160 μmol · m^{-2} · sec^{-1} at 25°C.

Dilution methods were applied for the isolation of a picoplanktonic species. The species should be maintained by axenic culture. However, it is too difficult to persist axenic culture in this experimental condition. Therefore, a unialgal culture system was maintained.

Ultrastructure observations

To investigate the ultrastructure of the isolated picophytoplanktonic cells, samples were flowed through a membrane filter (0.2 μm pore size, Whatman) and then the filter with cells was transferred to vial containing 3% glutaraldehyde(GA) in filtered seawater and fixed in the solution for 90 minutes at room temperature. After fixation in 3% GA, cells on the filter were rinsed in wash solution with decreasing seawater concentration and finally with double distilled water. Before postfixation, cells were embedded in molten (60°C) agar (4%) and quickly cooled. The embedded agar was cut into small agar cubes. Small agar cubes were postfixed in 1% osmium tetraoxide(OsO4) solution for one hour at room temperature and washed. Fixed specimens were dehydrated in a graded ethanol series to 95% at room temperature. The 95% ethanol was replaced by three changes of absolute alcohol and followed by propylene oxide before the agar cubes were embedded in epoxy resin (Spurr, 1969). They were replaced in a graded epoxy resin and cured in an oven at 70°C for eight hours. The resin blocks were cut with a glass or diamond knife on an ultramicrotome (LKB Nova) into thin sections, gray to silver section and mounted onto copper grids (400mesh Cu Grid). After sections staining with uranyl acetate and lead citrate, sections were examined and photographed with a JEOL JEM-1200EX/S TEM.

For observations under scanning electron microscope(SEM), samples collected on GF/F filters were fixed and dehydrated in a graded series of ethanol and followed by transition dehydrant, propylene oxide as in the TEM specimen preparation method. After the final change in 100% propylene oxide, samples were replaced into 1:1 mixture (one part propylene oxide: one part Peldri II) for one hour and the mixture was changed to 100% Peldri II for one hour. To allow the liquid Peldri II to solidity, samples submerged in Peldri II were cooled to below 23°C and maintained under this condition until the Peldri II was completely sublimed. After the Peldri II had been completely sublimed, the samples were coated with gold in a sputter coater prior to examination under the SEM and observed with a JEOL JSM 5400 SEM.

Pigment analysis

To measure chlorophyll contents, algal specimens

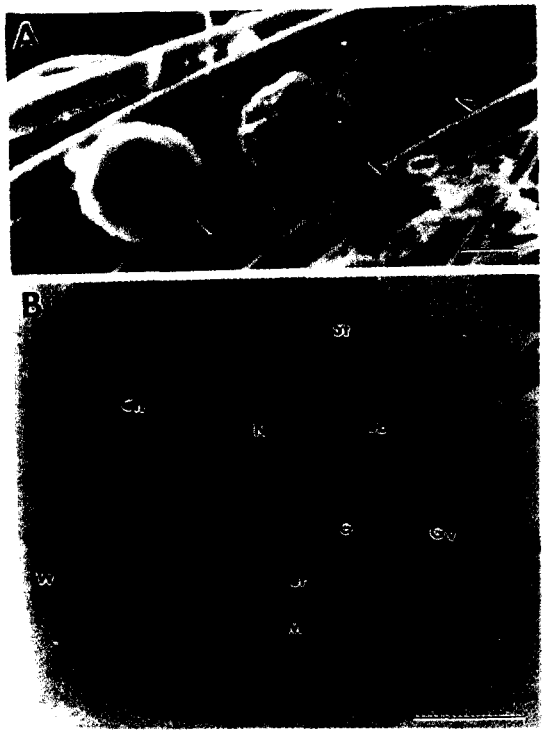

PLATE I

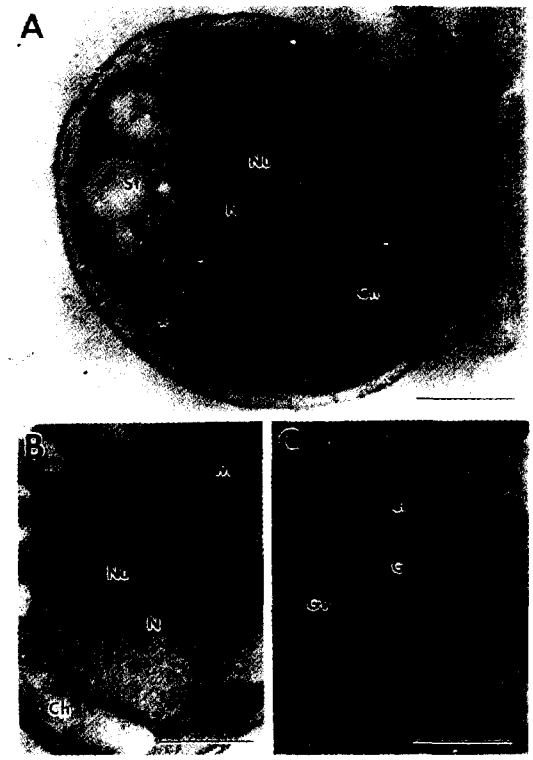

PLATE II

were filtered onto a membrane filter (0.45 μm pore size, Whatman) with two to three drops of $MgCO_3$ added to prevent degradation of chlorophyll. Filters with cells were subsequently extracted with 90% acetone, and chlorophyll contents were estimated with the spectrophotometric method (Parsons *et al.*, 1985).

Pigment analysis of the isolated pico-phytoplanktonic species was performed by HPLC. Algal specimens were collected through filtration onto Whatman GF/C filters using vaccum. The filters were extracted in 100% acetone. The filter debris and cell debris were almost removed by centrifuging for ten minutes at 10000 g at 4°C. All operations were performed in subdued light to avoid degradation of algal pigments.

The instrument used for HPLC was LC module I (Waters, Millipore Co., U.S.A). The solvent consisted of 90% acetonitrile (solution A) and ethyl acetate (solution B). The pigments were eluted using a linear gradient from 80% solution A to 20% solution B over thirty five minutes with a flow-rate of 1 $ml \cdot min^{-1}$ at room temperature. This solvent was pumped through Non-Encapped Zorbox ODS column (4.6×250 mm, 5 μM particle size) and 25 μl of the pigment was injected. Pigments were detected with an absorbance detector at 445nm.

RESEULTS AND DISCUSSION

Field study

The chlorophyll contents were ranged 0.01~0.05 μg chl $a \cdot l^{-1}$, which was put into oligotrophic condition. The nano-sized phytoplankton fraction consisted over 60% of total biomass but pico-sized phytoplankton showed below detection limit. Also, the effective grazing may cause near zero concentration of pico-sized phytoplankton.

Identification of the isolated algal specimen

The scanning electron micrograph of the isolated specimen showed that it was spherically shaped.

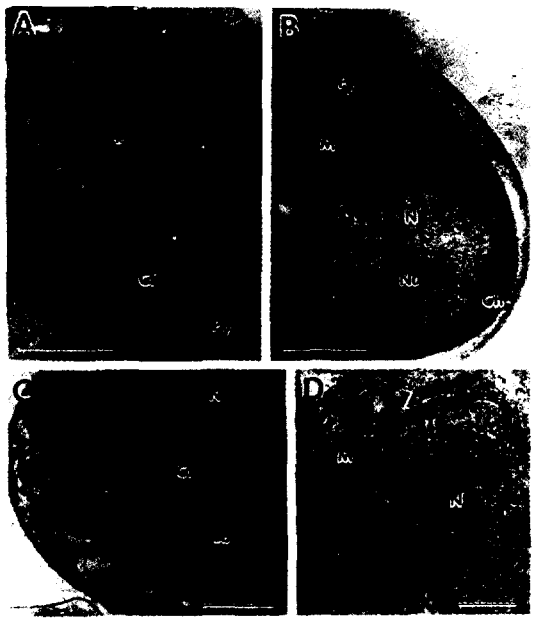

PLATE III

The specimen did not have a flagellum nor scales (Plate 1, Fig. A).

The transmission electron micrographs of thin sections of this alga also showed that the cell contained a nucleus, two to three chloroplasts, a mitochondrion, a Golgi body, Golgi vesicles, vacuoles, cell wall, endoplasmic reticula, lipid bodies and pyrenoids. The average size of the thin sections was 1.90 μm × 2.24 μm (Plates I, II and III).

The isolated species is a yellow-green colored and about 2 μm sized alga. Although this alga may be lain at the border line of picoplankton and ultraplankton in terms of size, it may be speculated that natural size may be smaller in the field than that isolated and cultured in the laboratory. The result of the analysis for the ultrastructure of the isolated species showed that it was a spherical picophytoplankton having similar thylakoid arrangement in chloroplast to Chlorophyta. The two to three chloroplasts were observed in the sections (Plate I, Fig. B; Plate II, Fig. A; Plate III, Fig. B). The chloroplasts contained two layered thylakoids and starch (Plate I, Fig. B; Plate II, Fig. A) as a reserve product and were surrounded by a chloroplast envelope unit (Plate III, Fig. D). A large nucleus was surrounded by a pored-nuclear envelope (Plate I, Fig. B; Plate II, Figs. A and B; Plate III, Figs. B and D). A nucleolus was identified in nucleus (Plate II, Figs. A and B). They also possessed pyrenoids (Plate III, Fig. B). A mitochondrion was identified near the nucleus (Plate III, Figs. B and D). There were a lot of vacuoles within the cell. Amorphous materials in the vacuole near plasma membrane were shown (Plate II, Fig. A). The protoplasm was distributed evenly as particle forms. Cell division by furrow was occasionally identified through a cleavage furrow (Plate III, Figs. A and C). Unidentified multi-layered structures were recognized in dividing cells (Plate III, Fig. A).

As shown in the ultrastructure of the alga, it contains nuclear envelope, a mitochondrion, a Golgi body, endoplasmic reticulum and chloroplasts. The alga belongs to an eukaryotic phototroph.

Based on the following features, this alga belongs to Chlorophyta. Thylakoids seemed to be associated in two layers in the chloroplast. The chloroplast envelope showed unit membrane. There was not any chloroplast endoplasmic reticulum(CER) which is connected to the nuclear envelope and surrounds the chloroplast (Plate III, Fig. D). The divisions which do not have CER are Chlorophyta and Rhodophyta. Rhodophyta, however, has isolated thylakoid arrangement in chloroplast and this specimen has the chloroplast which contains two-layered thylakoid membranes. Consequently, the alga could be identified as a chlorophyte.

Among non-flagellated chlorophytes, the green alga appears to be a species of *Nannochloris*-like cells or *Chlorella* with 2~3 μm spherical cell by taxonomic keys (Johnson and Sieburth, 1982). The present alga does not seem to be a species of *Nannochloris* but *Chlorella*. The cell do not have multilayered cell wall which is the characteristic of *Nannochloris* but single-layered cell wall as *Chlorella*.

Chlorella minutisima seems to be the most similar species in terms of size (1.8~3.0 μm). The feature of nucleus and the position of the pyrenoid situated in the 'hollow' of the cup-shaped chloroplast in *C. minutisima* were similar to this specimen. However, it has a single chloroplast and the pyre-

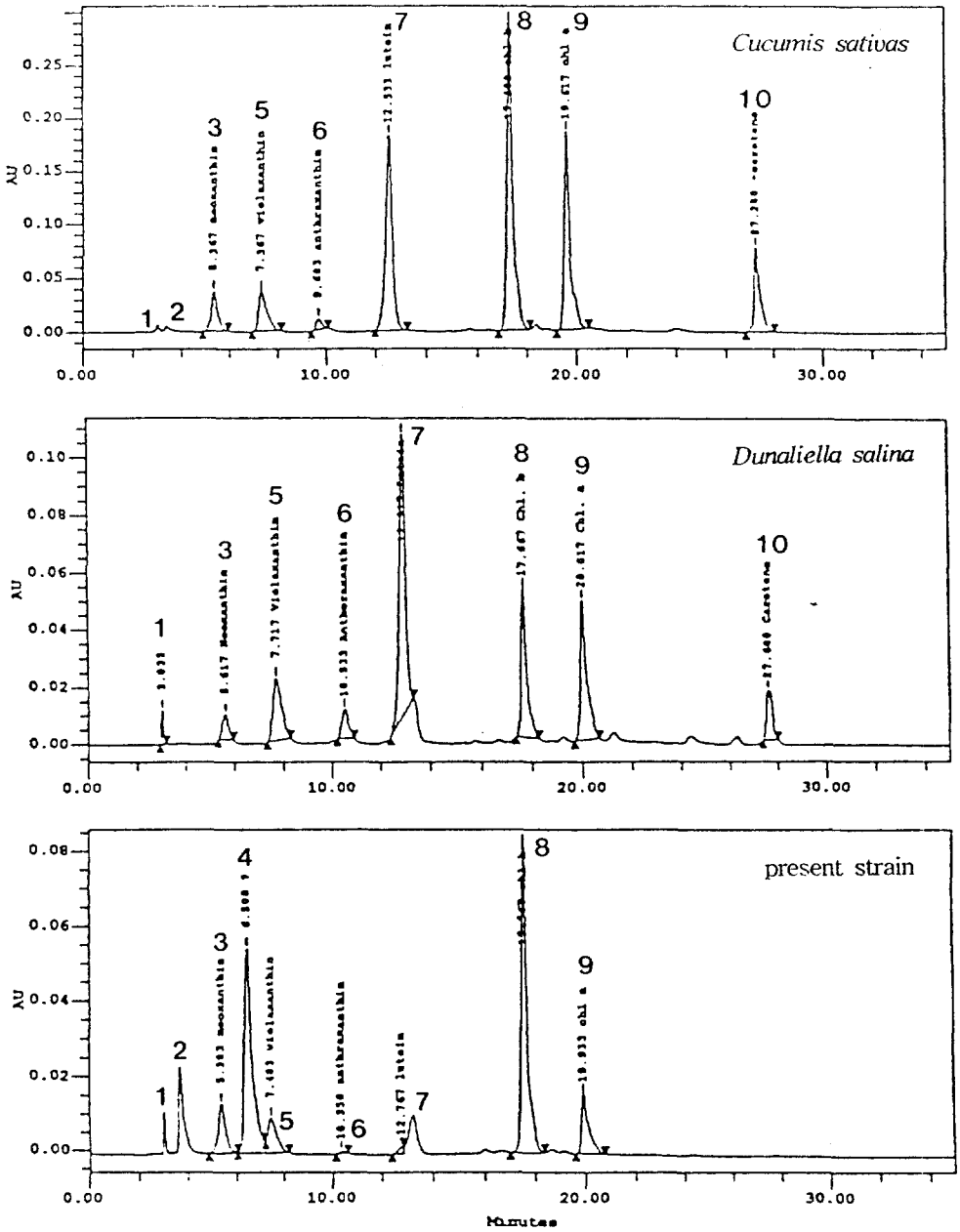

Fig. 2. The chromatographs of *Cucumis sativas*, *Dunaliella salina* and the isolated picoplankton by HPLC. (1. unknown; 2. unknown; 3. neoxanthin' 4. z-isomer of violaxanthin; 5. violaxanthin; 6. anthraxanthin; 7. lutein; 8. chlorophyll *b*; 9. chlorophyll *a*; 10. β-carotene).

noid of the species was always surrounded by a continuous layer of starch. *C. minutisima* has a tubular, high branched mitochondrion around a chloroplast (Dempsey *et al.*, 1980). Most of all, this species is a freshwater species. The isolated species could not be considered as *C. minutisima*, but the possibility of the appearance of the freshwater species in marine environment could not be excluded.

The isolated specimen could be regarded as *Chlorella nana*, which has appeared at the Northern Adriatic Sea (Andreoli *et al.*, 1978). *C. nana* is spherical, 1.5~3.0 μm sized. It is very similar to the isolated alga. *C. nana,* however, has no pyrenoid.

There might be also a very strong another possibility that the isolated species might be a prasinophyte. The isolated picophytoplankton was very similar to an unidentified prasinophyte found from the Coral Sea in Australia by Clayton and King (1990) in the pyrenoid structure, two to three peripherical chloroplasts and nucleus structure. However, the motile organs such as flagella, basal bodies and scales which covered the surface were not shown in this isolated picophytoplankton. Therefore, it is difficult to say that it is a species of prasinophyte. In comparison of the ultrastructure of both transmission electron micrographs, however, the both species showed very similar appearance.

In further study, serial sections are minutely required to identify the species. Serial sections can show the three-dimensional arrangement of organelles (Dempsey *et al.*, 1980). For detailed ultrastructural study of the isolated specimen, the examination through a serial section should be carried out.

Pigment analysis

To confirm the fact that the isolated specimen is a green alga, HPLC analysis was performed. For this attempt, the chromatographs of other green alga and a higher plant were examined as reference specimens. Chromatographs used in comparison were these of pigment extracts from a green alga, *Dunaliella salina* (Calorina Biol. Suppl. No. 15-2160) and a higher plant, cucumber (*Cucumis sativas* L. cv. *Ilmichungjang*). Chromatographs of pigment extracts from these specimens were shown in Fig. 2.

As a result, it showed that the species contained the same pigment composition as shown in *D. salina* and cucumber, which had pigments such as chlorophyll *a*, chlorophyll *b*, neoxanthin, violaxanthin, lutein, anthraxanthin and β-carotene. The certain peaks distinguished from the pigment extracts from both species were also observed before the violaxanthin was seperated. It seems to be an unidentified Z-isomer of violaxanthin. Chlorophyll *a* to *b* ratio was higher than that of both species. The peaks of β-carotene were particularly lower than other two species (data not shown).

In order to prove clearly the presence of chlorophyll *b*, the pure chlorophyll *b* was added to the pigment extract from the isolated alga. The enhancement of the peak of chlorophyll *b* was observed (data not shown) and the presence of chlorophyll *b* was verified. Through the HPLC analysis with the ultrastructural observations, consequently, the species was identified as Chlorophyta. In further study, the analyses of other significant accessary pigments and carotenoids are required for species identification of the isolated species.

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