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A RAPID AND CONVENIENT METHOD FOR PURIFICATION AND ISOLATION OF CHLOROPHYLL-A FROM *PORPHYRA YEZOENSIS*

Running title: PREPARATION OF CHLOROPHYLL-A

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SUMMARY: A rapid and convenient method for purification and isolation of chlorophyll-a from *Porphyra yezoensis* without any chromatographic procedures has been developed. Thin-layer chromatographic and high-performance liquid chromatographic tests revealed that chlorophyll-a preparations in this study did not contain any other photosynthetic pigments and their degradation products.

INTRODUCTION: Since many investigators have been researching to elucidate the function of chlorophyll molecules on a close comparison of *in vitro* systems with intact *in vivo* preparations or even whole cells, needs for highly purified chlorophyll preparations are now increasing in various scientific fields. Column chromatography is a common method to prepare chlorophylls in quantities of 0.1-1 g. However, we experienced some difficulties in separating photosynthetic pigments from each other by column chromatography. Therefore, we aimed to develop a method for preparation of chlorophyll-a without any chromatographic procedures. Recently, we have developed a method for partial purification of chlorophylls extracted from spinach leaves using of dioxane (1), and washing 80 % (v/v) aqueous methanol (2) before chromatography. Blue-green and red algae may be essentially effective as starting materials to prepare pure chlorophyll-a because these algae have only chlorophyll-a as photosynthetic green pigment. Hence, red alga, *Porphyra yezoensis*, was choiced

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as one of the starting materials to prepare chlorophyll-a as a preliminary study, and a good result was obtained. In the present communication, a method for the preparation of chlorophyll-a will be reported.

MATERIALS AND METHODS: *Porphyra yezoensis* was bought at a market near Kamakura in Japan. All the solvents used were of analytical reagent grade and were used without further purification, unless otherwise mentioned. The powdered sugar used was standard confectioner's sucrose containing 3 %, by weight, of corn starch. The powdered sugar was dried over night in an oven at 80°C. All other chemicals were also of analytical reagent grade and were used without further purification. Absorption spectra of pigments were measured with a Hitachi ESP-3T spectrophotometer. Pigments were characterized by comparison of their visible spectra with literature values. In addition, the purity and stability of chlorophyll-a molecules were examined by thin-layer chromatography (3), and high-performance liquid chromatography (4,5), as it has been already described (6) and this author also has accepted that the spectroscopic observation should be supplimented by chromatographic tests to demonstrate that chlorophyll-a molecules were not altered. It was possible to detect qualitatively the pigments to the order of 10^{-8}g and 10^{-10}g by using of the thin-layer chromatographic method (3) and the high-performance liquid chromatographic method (4,5), respectively.

RESULTS: All the experiments in this study were carried out at about 5°C in total darkness or under dim green light, unless otherwise mentioned.

20 g of fresh *Porphyra yezoensis* were homogenized for 3 min in a Waring blendor with 100 ml of acetone. The green juice obtained were filtered through a Buchner funnel to remove coarse debris and the filtrate was centrifuged at 10,000xg for 5 min to remove insoluble materials. The deep green supernatant solution thus obtained was mixed with 14 ml dioxane and then 15-20 ml of 10^{-2}M pH 8 phosphate buffer were added drop-wise, with stirring, until high turbidity developed to precipitate chlorophyll-a--dioxane adducts as a radial crystalline form (see Fig.1 (d) in ref. 1). The solution was placed in an ice box for one hour to allow sedimentation. The top portion was decanted and the lower

thick mass of dark green sediment was collected by centrifugation at 10,000xg for 5 min. The crude mass of chlorophyll-a thus obtained (Ppt I) was dissolved in about 30 ml of acetone : dioxane mixture (7 : 1, v/v) and precipitated again by drop-wise addition of about 4-5 ml of distilled water. The crude mass of chlorophyll-a prepared by the second dioxane treatment (Ppt II) was collected by the centrifugation and was evaporated to dryness under reduced pressure. Ppt II was dissolved into 100 ml of methanol containing 25 ml of petroleum ether (b.p. 20-40°C) and then 50 ml of distilled water was added into the solution. The upper petroleum ether layer was washed with 80 % (v/v) aqueous methanol repeatedly until the lower aqueous methanol layer lost yellowish color completely. The petroleum ether solution was evaporated under reduced pressure and was dried in a vacuum desiccator, and then the dark-green micro-crystals (Ppt III) were obtained as a resultant. Ppt III was suspended into 5-10 ml of petroleum ether containing trace diethyl ether (less than 1 % (v/v)) and the suspension was filtered through a powdered sugar layer (4 cm in height and 3 cm in a diameter) packed in a glass funnel with fritted disc #3, which was connected to a suction bottle. Chlorophyll-a molecules were adsorbed in the powdered sugar layer and nonsorbed carotenes removed from the sugar layer by washing with petroleum ether. After washing out the nonsorbed carotenes from the sugar layer, chlorophyll-a was extracted from the sugar layer with diethyl ether. Thin-layer chromatographic analysis of the chlorophyll-a preparation revealed that the preparation contained neither carotenoids nor degradation products of chlorophyll-a. Analysis of the preparation in the high-performance liquid chtomatography also supported the thin-layer chromatographic observation. Absorption spectra of the chlorophyll-a preparations dissolved in diethyl ether did not show any significant differences in comparing with the literature within experimental error; the ratio of absorbance of the Soret peak at 427.0 (± 1.0) nm to the red peak at 660.0 (± 0.5) nm was 1.30 (± 0.01).

DISCUSSION: The solvent most effective for the extraction of chlorophyll-a from the red alga was 90 % (v/v) aqueous methanol. However, it was observed for some times the formation of allomerized chlorophyll-a (7) as a minor component in the methanol extracts. Therefore, 90 % (v/v) aqueous acetone was used in this study to extract chlorophyll-a from the material. When the acetone extracts were kept for a long periods (more than 2 hrs), chlorophyll-a molecules in the solutions were converting to pheophytin-a, chlorophyll-a′, chlorophyllide-a, and any other degradation products of chlorophyll-a. Hence, all the procedures for the preparation of chlorophyll-a were accomplished smoothly and swiftly to obtain highly purified chlorophyll-a preparations. Dioxane Method (1) is principally based on the fact (8,9) that dioxane interacts with chlorophyll-a to form chlorophyll-a-dioxane adducts selectively and that the adducts are precipitated in microcrystalline form, leaving the bulk carotenoids in solution. The procedure of washing Ppt II with 80 % (v/v) aqueous methanol was essentially neccessary to remove the resultant carotenoids except for the nonsorbed carotenes in Ppt II after dioxane treatment. *Anacystis nidulans* was also examined to prepare chlorophyll-a, and a good result was obtained by the same procedures.

REFERENCES:

1. Iriyama, K., Ogura, N., and Takamiya, A. (1974) J. Biochem. (Tokyo) 76, 901-904.

2. Iriyama, K., Shiraki, M., and Yoshiura, M. (1977) Chem. Lett. (Tokyo) pp. 787-788.
3. Shiraki, M., Yoshiura, M., and Iriyama, K. (1978) Chem. Lett. (Tokyo) pp. 103-104.
4. Yoshiura, M., Iriyama, K., and Shiraki, M. (1978) Chem. Lett. (Tokyo) pp. 281-282.
5. Iriyama, K., Yoshiura, M., and Shiraki, M. J. Chromatogr. in the press.
6. Strain, H.H., and Svec, W.A. (1966) The Chlorophylls, pp. 21-66, eds. Vernon, L.P., and Seely, G.R., Academic Press, New York.
7. Johnston, L.G., and Watson, W.F. (1956) J. Chem. Soc. pp. 1203-1212.
8. Iriyama, K., Yoshiura, M., and Asai, H. (1974) Chem. Lett. (Tokyo) pp. 787-790.
9. Iriyama, K., and Yoshiura, M. (1977) J. Colloid and Polymer Sci. 255, 133-139.