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Improved Separation Method for Highly Purified Lutein from *Chlorella* Powder Using Jet Mill and Flash Column Chromatography on Silica Gel

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We investigated an improved method for the separation of high-purified lutein from a commercially available spray-dried *Chlorella* powder (CP) using fine grinding by jet mill and flash column chromatography on a silica gel. Saponification and extraction of lutein were enhanced 2.3−2.9-fold in jet mill-treated CP (mean particle size, 20 μm) as compared to untreated CP (mean particle size, 67 μm). The carotenoid extract was dissolved in ether−hexane (1:1 v/v) and subjected to flash column chromatography on silica gel. A mixture of α- and β-carotene was eluted with hexane, followed by elution with hexane−acetone−chloroform (7:2:1 v/v). Lutein (dark-orange band) was collected after the elution of an unknown colorless compound (detected based on UV absorbance). The purity of lutein in this fraction was over 99%, and the yield was 60%. The present study provides key information for obtaining highly purified lutein using flash column chromatography on a silica gel.

KEYWORDS: Lutein; *Chlorella*; jet mill; silica gel; column chromatography

INTRODUCTION

Lutein is a carotenoid abundant in vegetables, fruits, and green plants. Recently, the nutritional and biological importance of lutein has been reported. Some epidemiological studies have shown that lutein is effective in the prevention of coronary heart disease, cancer, age-related macular disease, and cataracts (*1*). Lutein is the predominant carotenoid in the macular pigment (*2*, *3*) and may prevent damage from light in macular cells. Proposed functions of lutein, which maintains the health of the eye, are an antioxidative effect involving the quenching of singlet oxygen and absorption of the blue wavelength in light.

It has been documented that *Chlorella* has biological effects in animal and human studies (*4*−*7*). Also, *Chlorella* is known as a green microalga containing large amounts of carotenoids (*8*). A strain of *Chlorella* was isolated that can be grown under heterotrophic conditions with a high growth rate (*9*) and has enabled the industrial production of *Chlorella* in a stealized fermentor (under dark conditions) without contamination. Zeaxanthin is a stereoisomer of lutein widely contained in green plants and flowers. Zeaxanthin is a main contaminant of highly purified lutein, and it is very difficult to separate lutein from zeaxanthin. Thus, it is of importance that a strain of *Chlorella* does not accumulate zeaxanthin. Several methods for the isolation and purification of lutein from *Chlorella* have been reported (*8*, *10*). Column chromatography using alumina as an adsorbent for identification (*8*) and a two-phase system for removing the fat and water-soluble impurities of the pigment extract of *Chlorella* (*10*) were developed. In general, commercially available CP is spray-dried. However, extraction and purification studies were carried out with freeze-dried *Chlorella* (*10*) or viable *Chlorella* cells (*8*).

TLC and semipreparative HPLC have a high resolution for the isolation and purification of lutein; however, the amount of lutein obtained by these methods is very small. Column chromatography is often used for the purification of organic chemicals in the laboratory and also on an industrial scale. In normal phase column chromatography, silica gel is widely used as the stationary phase because of its high resolution and economical performance. At present, column chromatography on a mixture of silica gel and diatomaceous earth is used for the quantification of xanthophylls (*11*); however, there is no published study describing the preparative isolation of highly purified lutein using flash column chromatography on silica gel. Thus, no information is available as to the gram scale preparation of lutein with high purity, which can be used as a reagent for biological and chemical studies.

The aim of this study was to develop a simple and efficient method for the preparative separation of lutein with high purity from spray-dried CP (*Chlorella regularis*) using flash column chromatography on silica gel. Moreover, the effect of fine grinding of spray-dried CP by jet mill treatment on the saponification and extraction of lutein is discussed.

MATERIALS AND METHODS

Materials. Spray-dried *Chlorella* powder (CP) was obtained from Nihon Chlorella Co. Ltd (Tokyo, Japan). The *Chlorella* contained 2.50 mg of lutein (per g of dried *Chlorella*). Standard α-carotene, β-carotene, and lutein were purchased from Sigma Chemical Co. (St. Louis, MO). Silica gel was obtained from Fuji Silysia Chemical Ltd. (FL60D, particle

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size, 60 μm, Aichi, Japan). The glass column for flash column chromatography was purchased from Kimura Scientific Glass Co., Ltd. (Tokyo, Japan). The methanol, acetonitrile, and tetrahydrofran were HPLC grade (Junsei Chemical Co., Ltd., Tokyo, Japan), and all other reagents were analytical grade (Wako Pure Chemical Ind., Osaka, Japan).

Fine Grinding of CP by Jet Mill Treatment. Fine grindinig of CP was done with a vortex flow jet mill (single track jet mill, Model STJ-200, Seishin Enterprise Co., Ltd., Tokyo, Japan). Particle size was determined by the method of laser diffraction (SK Laser Micron Sizer, Model Pro-7000S, Seishin Enterprise Co., Ltd., Tokyo, Japan).

Saponification and Extraction of Crude Carotenoids. CP (40 g) was added to 400 mL of alkaline ethanol (6% KOH), and then the mixture was heated at 50 °C for 30 min. On being cooled to room temperature, the mixture was filtered through paper filter No.7 (Kiriyama-Seisakusho Ltd., Tokyo, Japan) to obtain the pigment solution. The solution was concentrated to about 150 mL in a rotary evaporator. Two hundred milliliters of ether and 250 mL of Na_2SO_4 anhydride solution (5%) were added to the pigment solution. The upper phase (ether fraction) was collected as a crude carotenoid solution, and then water-soluble impurities were extracted with water until the water phase was colorless.

Flash Column Chromatography on Silica Gel. Flash column chromatography was performed with a PUMP 540 and PREP UV-10V (Yamazen Corp., Osaka, Japan). The crude carotenoid solution was concentrated to a yellow-orange residue using a rotary evaporator. This residue was dissolved with 100 mL of ether, and an equal volume of hexane was added. After filtration, this solution was subjected to flash column chromatography (30 mm i.d. × 260 mm) on silica gel, equilibrated with hexane. The eluent flowed through the column under a pressure of 1−2 kg/cm^2. At first, the column was eluted with hexane to afford a mixture of α- and β-carotene. When elution of the carotene band was completed, the eluent was changed to hexane−acetone−chloroform (7:2:1 v/v). Fractions 1−3 were collected based on UV absorbance (fraction 1) or band of color (yellow−orange, fractions 2 and 3).

Determination of Carotenoids by HPLC. The HPLC analysis was performed with a 600E pump (Waters, Milford, MA), 991J photodiode array detector (Waters), and 700 Satellite WISP autosampler (Waters). Data acquisition and quantification were made on a Millenium chromatography data system (Waters). The effluent was monitored from 200 to 700 nm, and the carotenoids were detected at 450 nm. The column used was a YMC-Pack ODS-A 302 column (YMC, Kyoto, Japan; particle size, 5μm; column size, 4.6 mm i.d. ×150 mm). The mobile phase consisted of methanol−acetonitrile−tetrahydrofran (45:50:5 v/v). The flow rate was set at 1.0 mL/min, and the column was kept at 30 °C. The injection volume was 10 μL.

Identification of Lutein Using Absorption Spectra. Pigment solutions fractionated by flash column chromatography were evaporated under a nitrogen stream and then dissolved in ethanol. Their spectra were obtained with a UV−vis recording spectrophotometer (UV-160A, Shimadzu Co., Kyoto, Japan).

Statistical Analysis. A Tukey's multiple comparison test (SAS system version 8.2, SAS Institute Inc., Cary, NC) was used to determine the differences between the extraction of lutein from CP, jet mill treated CP, and freeze-dried CP.

RESULTS AND DISCUSSION

Effect of Fine Grinding of CP by Jet Mill on the Extraction of Carotenoids. The identification and quantification of carotenoids contained in *Chlorella regularis* has been reported (*12*). The major components are two carotenes (α- and β-carotene) and three xanthophylls (lutein, violaxanthin, and neoxanthin). A typical HPLC chromatogram of a crude carotenoid solution of CP is shown in **Figure 1**. Lutein, α-carotene, and β-carotene were clearly separated and identified using the retention time of each standard (**Figure 1b**). Two unidentified peaks (unidentified 1 and 2) were present before and after the lutein peak. We have not identified these peaks but presumed them to be violaxanthin and neoxanthin because they have similar chemical properties to xanthophylls (oxygenated carotenoids). There was another unidentified peak present in the chromatogram at 280 nm (unidentified 3, **Figure 1a**).

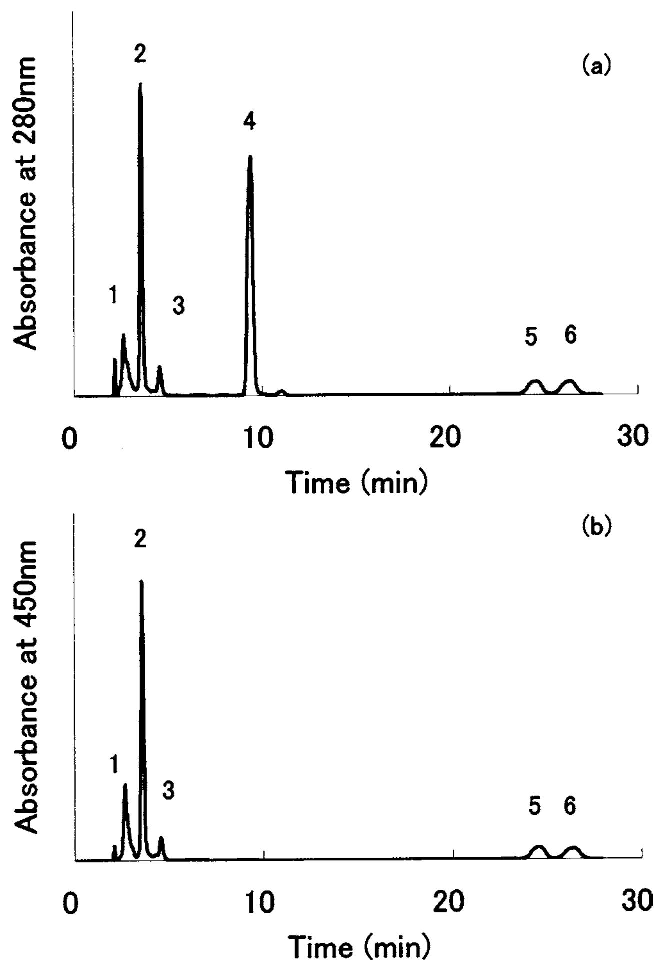

Figure 1. HPLC chromatogram of crude carotenoid solution from CP. (**a**) Absorbance at 280 nm. (**b**) Absorbance at 450 nm; peak 1: unidentified 1, peak 2: lutein, peak 3: unidentified 2, peak 4: unidentified 3, peak 5: α-carotene, and peak 6: β-carotene.

Table 1. Extraction of Lutein from CP, Fine-Ground CP, and Freeze-Dried *Chlorella* Cells after Saponification

	CP[a] (untreated)	CP[b] (jet mill treatment)	freeze-dried *Chlorella* cells
extraction of lutein (mg of lutein/g of dried *Chlorella*)	0.50 ± 0.06[c]	1.46 ± 0.20[d]	0.76 ± 0.08[c]

[a] The mean particle size was 67 μm. [b] The mean particle size was 20 μm. [c,d] Data are presented as mean ± SD for three independent experiments. Means in columns followed by different letters were significantly different, by Tukey's multiple comparison test ($p < 0.05$).

As can be seen in **Table 1**, the yield of lutein from CP in the extraction step was about 20% (CP contained 2.50 mg of lutein/g of CP). One potential cause of the low yield is that *Chlorella* cells are aggregated in particles of CP because the CP was manufactured with a spray-drier. Moreover, saponification and extraction were not carried out with a mechanical grinder such as a blender or homogenizer. Thus, the effects of fine grinding with a jet mill or freeze-drying of CP on the extraction of lutein were investigated. Jet mills are widely used in various industries

Table 2. Effect of the Particle Size of JMC on the Extraction of Carotenoids

mean particle size of CP (μm)	relative contents of each carotenoid extracted from CP or JMC of different particle sizes: untreated CP 67	JMC 39	JMC 20	JMC 10	JMC 5
lutein	100[a]	180	232	284	304
α-carotene	100	188	260	362	429
β-carotene	100	186	254	342	413
unidentified 1	100	227	362	547	704
unidentified 2	100	274	492	809	1104

[a] Values are normalized to untreated CP (100). Data are presented as mean values of two independent experiments.

to obtain finely ground powders. From a chemical point of view, a jet mill is suitable for grinding heat-sensitive material. This is one of the reasons for choosing this milling method because lutein is sensitive to heat, light, and oxygen.

The mean particle size of CP is 67 μm, and jet mill treatment decreased this to 20 μm. As shown in **Table 1**, the jet mill treatment had a strong influence on the extraction of lutein. The amount of lutein extracted from jet mill treated CP (JMC) was about three times that from untreated CP. On the other hand, the amount of lutein extracted from a freeze-dried *Chlorella* cell was not significantly different from that from spray-dried CP.

Next, we focused on the effect of particle size on the extraction of carotenoids (**Table 2**). As shown in **Table 2**, there was an inverse relationship between the amount of carotenoid extracted and the particle size of JMC. The enhancement of the extraction of lutein by jet mill treatment is considered to be caused by (1) disruption of *Chlorella* cells and (2) extension of the surface area with a decrease in particle size. The results described previously are also explained in part by the effect of particle size (surface area); however, the effect of the disruption of *Chlorella* cells requires more study using a scanning electron microscope.

Interestingly, the rates of increase in the extraction of each carotenoid differed. Notably, unidentified 1 and 2 were markedly increased. The reason for these differences is unclear. In the separation of lutein by flash column chromatography on silica gel, we used JMC, the mean particle size of which was 20 μm, as a starting material.

Flash Column Chromatography on Silica Gel To Obtain Highly Purified Lutein from Crude Carotenoid Solution. Matsuno reported the separation of carotenoids from *Paralithodes herevipes* using silica gel chromatography (*13*). In that report, β-carotene was eluted with hexane and xanthophylls (containing lutein) with ether. A silica gel-packed column was used for the separation of leaf carotenoids with a gradient of hexane-propan-2-ol solvent (*14*). In the present study, we developed a solvent system for the isocratic elution of highly purified lutein on silica gel because an isocratic elution is simple and reproducible. The charge solution, in which final crude carotenoids from CP were dissolved, used for flash column chromatography consisted of hexane−ether (1:1 v/v). Under these conditions, lutein was adsorbed by the silica gel. After elution of the carotene mixture with hexane, the eluent was changed to solvent system 1; hexane−acetone−chloroform (7:2:1 v/v) and fractions 1−3 were collected based on UV absorbance (fraction 1) or bands of color (fractions 2 and 3). HPLC chromatograms of fractions 1−3 are shown in **Figure 2**. Fraction 1 (**Figure 2a**) was the unknown colorless compound

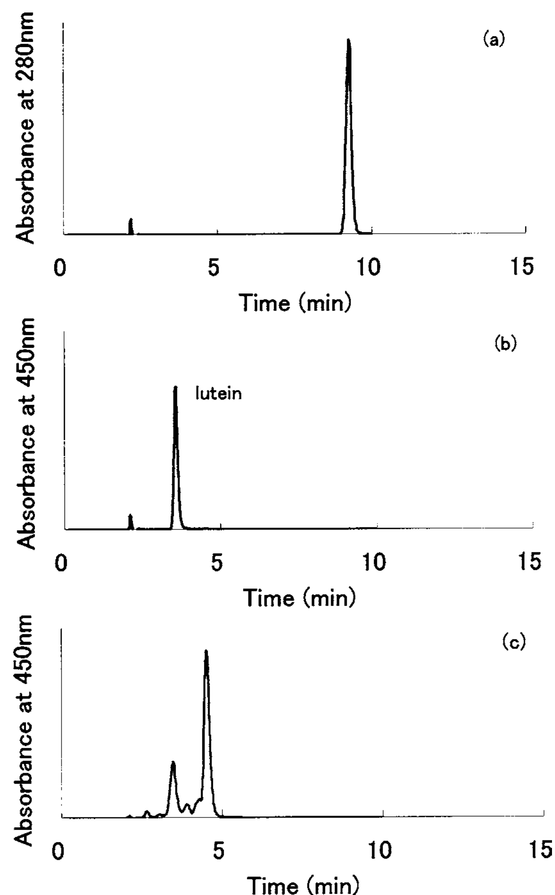

Figure 2. HPLC chromatogram of fractions 1–3. (**a**) Fraction 1 (unidentified 3, at 280 nm), (**b**) fraction 2 (lutein, at 450 nm), and (**c**) fraction 3 (unidentified 1 and 2, at 450 nm).

(unidentified 3 in **Figure 1a**) that was not detected at 450 nm and displayed a strong absorbance in the UV range. Fraction 2 was the first yellow−orange band eluted with solvent system 1. It is clear from **Figure 2b** that fraction 2 contained only lutein. The purity of lutein was over 99% by HPLC analysis. The clarified absorption maxima of fraction 2 were at 446 and 474 nm in ethanol. The reported data for visible spectra of lutein in ethanol was consistent with our data (*15*). Fraction 3 mainly consisted of unidentified 1 and 2. Interestingly, unidentified 1 was eluted after lutein, as compared to the ODS−HPLC system.

The final yield of lutein in fraction 2 was 60%. To clarify the possibility that lutein was adsorbed by the silica gel, 100 mg of lutein was subjected to flash silica gel (100 g) chromatography, and the recovery of lutein was measured. The rate of recovery was almost 100%. Thus, the combination of silica gel and solvent system 1 is very efficient for the purification of lutein, and the key steps determining yield in the present study were the extraction and partition. To improve the yield of lutein, optimization of the extraction and partition steps is needed.

A method of purifying lutein from saponified marigold oleoresin (SMO) with recrystallization has been reported (*16*). Recrystallization is an efficient method for the isolation and purification of SMO because lutein accounts for 90% of the carotenoids in SMO. However, it is a time-consuming process, and its recovery rate decreases with the increased purity of lutein. In contrast, our method can be applied to the purification of lutein from SMO to obtain a final purity over 99%. Also, the method presented in this paper can be adapted to the isolation and purification of lutein from green vegetables such as kale and spinach.

The main advantage of flash column chromatography on silica gel is the ease with which the process can be scaled up. Actually, the present method was applied on the following scale: JMC (starting material), 5 kg; reactor (saponification and extraction), 60 L; column chromatography, 29 cm i.d. × 100 cm; and silica gel, 10 kg. The amount of lutein obtained was approximately 6 g, and the purity was 99% as determined by HPLC analysis. The final yield of lutein was approximately 50%.

In conclusion, fine grinding of CP using a jet mill is an effective pretreatment for the extraction of lutein. Flash column chromatography on silica gel is a simple, reproducible, economical, and preparative method of obtaining highly purified lutein from CP. The results of this study will contribute to the isolation and purification of lutein from natural sources of lutein such as SMO and green plants.

ABBREVIATIONS USED

CP, *Chlorella* powder; JMC, jet mill-treated CP; SMO, saponified marigold oleoresin.

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