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Preparative isolation and purification of astaxanthin from the microalga *Chlorococcum* sp. by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was applied to the isolation and purification of astaxanthin from microalgae. The crude astaxanthin was obtained by extraction with organic solvents after the astaxanthin esters were saponified. Preparative high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:6.5:3, v/v) was successfully performed yielding astaxanthin at 97% purity from 250 mg of the crude extract in a one-step separation.

Keywords: *Chlorococcum* sp.; Counter-current chromatography; Astaxanthin; Carotenoids; Antioxidants

1. Introduction

Numerous epidemiological studies in various populations have shown that the consumption of substantial amounts of fruits and vegetables reduce the risk of cancer. One hypothesis is that the carotenoids in these foods act as antioxidants through a free radical mechanism by quenching singlet oxygen and other oxidizing species resulting in the termination of free radical chain reactions and in the prevention of cellular oxidative damage. Carotenoids have been proposed as cancer prevention agents, life extenders, and the inhibitors of ulcer, heart attack and coronary artery disease [1–4]. The antioxidant activity of astaxanthin was 10 times stronger than that of other carotenoids, namely, zeaxanthin, lutein, canthaxanthin, and β-carotene [5]. Certain algae may serve as a continuous and reliable source of astaxanthin because they can be cultivated in bioreactors on a large scale [6–8]. Furthermore, the algal cells might contain no herbicides and pesticides, and any other toxic substances because the nutrient medium for growing the algae could be well formulated.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography that uses no solid support matrix. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. The method has been successfully applied to the analysis and separation of various natural products [9–14]. However, no report has been published on the use of high-speed counter-current chromatography for the isolation and purification of astaxanthin from micro-

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algae. The purpose of this study, therefore, was to develop a method for the isolation and purification of astaxanthin from the microalga *Chlorococcum* sp. by high-speed counter-current chromatography.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (diameter of tube, 2.6 mm; total volume, 325 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a Model Series II HPLC pump (Pharma-Tech Research), a Model SPD-10Avp UV–Vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10-ml sample loop.

The HPLC system used throughout this study consisted of a Waters 510 pump (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium chromatography data system (Waters). The column used was a reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm, Beckman, Fullerton, CA, USA).

2.2. Reagents

All solutions were prepared with analytical grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethanol, *n*-hexane, ethyl acetate, methanol, dichloromethane and acetonitrile were obtained from BDH (Poole, UK). The astaxanthin stock solution was 0.100 mg ml^{-1} which was prepared by dissolving 1.00 mg of astaxanthin (Sigma, USA) in 10.00 ml of the mobile phase for HPLC (dichloromethane–methanol–acetonitrile–water, 5:85:5.5:4.5, v/v) and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with the mobile phase. All other solutions were prepared by dissolving appropriate amounts of commercially available chemicals in water.

The microalga *Chlorococcum* sp. was obtained from our laboratory.

2.3. Preparation of crude astaxanthin from the microalga Chlorococcum sp.

Preparation of crude astaxanthin was carried out according to the literature [15]. In brief, 250 ml of 0.018 mol l^{-1} KOH solution was added into 100 g of the lyophilized alga, and the mixture was kept for 8 h under nitrogen in darkness at ambient temperature (22°C). A total of 50 ml of *n*-hexane–ethanol (1:1, v/v) was added to the mixture for the extraction of astaxanthin. The mixture was then separated by centrifugation at 10 000 *g* for 15 min, and the supernatant was collected. The extraction procedure was repeated until the cell debris was almost colorless, and all extracts were combined. Then, the extract was diluted to 1/2 with distilled water, and two phases were separated with a separatory funnel. The organic phase (*n*-hexane phase) was washed with 30% aqueous ethanol until the water phase was almost colorless and the pH was near neutral. After separation, the organic phase was blown to dryness under a stream of nitrogen, and the residue was stored in a refrigerator for the subsequent HSCCC separation.

2.4. Preparation of two-phase solvent system and sample solution

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the stationary phase of the solvent system used for separation.

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 2.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml of the sample solution containing 250 mg of the crude astaxanthin was injected through the injection value. The effluent of the column was continuously monitored with UV–Vis detection at 480 nm. Peak fractions were collected according to the elution profile.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC according to Yuan and Chen [16]. The mobile phase was solvent A (dichloromethane–methanol–acetonitrile–water, 5:85:5.5:4.5, v/v) and solvent B (dichloromethane–methanol–acetonitrile–water, 22:28:45.5:4.5, v/v) in gradient mode as follows: 0% of B for 8.0 min; a linear gradient from 0 to 100% of B for 6 min; 100% of B for 51 min. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 480 nm.

3. Results and discussion

Fig. 1 shows HPLC analysis of the astaxanthin crude from the microalga *Chlorococcum* sp. as well as the chemical structure of astaxanthin. Peak A corresponds to astaxanthin.

Preliminary high-speed counter-current chromatography studies were carried out with the two-phase solvent system composed of *n*-hexane–ethanol–water at various volume ratios (10:8:2, 10:8.5:1.5 and 10:9:1). The retention time of astaxanthin was too long with the two-phase solvent system at ratios 10:8:2 and 10:8.5:1.5. The retention of the stationary phase was small (about 15%) with the two-phase solvent system at ratios 10:9:1, and it was very difficult to separate astaxanthin from other substances. Thus, the two-phase solvent system composed of *n*-hexane–ethanol–water was not suitable

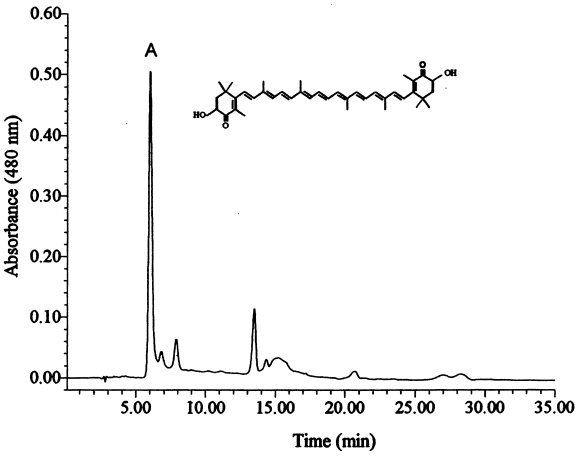

Fig. 1. Chromatogram of crude astaxanthin from the microalga *Chlorococcum* sp. by HPLC analysis as well as the chemical structure of astaxanthin; A, astaxanthin. Conditions: column, reversed-phase ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase, solvent A (dichloromethane–methanol–acetonitrile–water, 5:85:5.5:4.5, v/v) and solvent B (dichloromethane–methanol–acetonitrile–water, 22:28:45.5:4.5, v/v) in gradient mode; flow-rate, 1.0 ml min^{-1}; detection, 480 nm.

for the separation of astaxanthin from algae, although this two-phase solvent system was satisfactory for the separation of lutein from algae by HSCCC [9]. In subsequent studies, another two-phase solvent system was tested.

Performance of the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios (5:7:7:3, 5:5:7:3, 5:5:6.5:3.5 and 5:5:6.5:3) was evaluated in terms of peak resolution. The retention of the stationary phase was also small (about 20%) with the two-phase solvent system at ratio 5:7:7:3, and it was very difficult to separate astaxanthin from other substances. When the two-phase solvent system at ratio 5:5:7:3 was used, the retention of the stationary phase was better (about 60%), but, the peak resolution was not satisfactory (data not shown). The two-phase solvent system at ratio 5:5:6.5:3.5 was subsequently tested. The peak resolution was satisfactory, and it was found that the retention time was long (about 5 h). When the two-phase solvent system at ratio 5:5:6.5:3 was used, the peak resolution and the retention time were all satisfactory. Fig. 2 shows the preparative HSCCC separation of 250 mg of the crude sample using the solvent system composed of *n*-hexane–

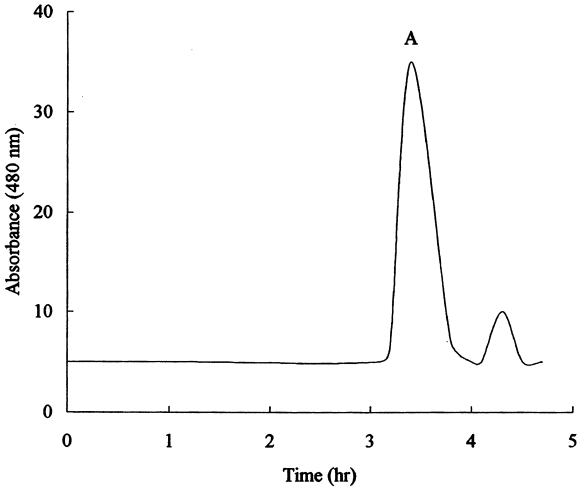

Fig. 2. Chromatogram of crude astaxanthin from the microalga *Chlorococcum* sp. by HSCCC separation, A = astaxanthin. Conditions: column, multilayer coil of 2.6 mm I.D. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–ethanol–water (5:5:6.5:3, v/v); mobile phase, lower phase (ethanol–water); flow-rate, 2 ml min^{-1}; detection, 480 nm; sample size, 250 mg; retention of the stationary phase, 62%.

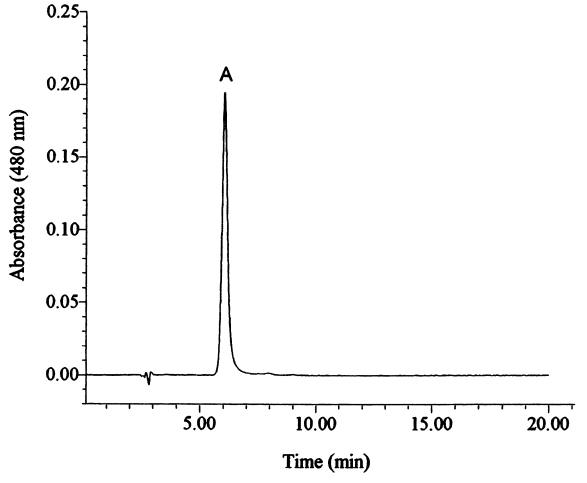

Fig. 3. HPLC chromatogram of astaxanthin purified from the microalga *Chlorococcum* sp. Conditions: column, reversed-phase ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase, solvent A (dichloromethane–methanol–acetonitrile–water, 5:85:5.5:4.5, v/v) and solvent B (dichloromethane–methanol–acetonitrile–water, 22:28:45.5:4.5, v/v) in gradient mode; flow-rate, 1.0 ml min^{-1}; detection, 480 nm.

Table 1
The *K* (partition coefficient) values of astaxanthin in several solvent systems

Solvent system	*K* value
n-Hexane–ethanol–water (10:9:1)	0.892
n-Hexane–ethanol–water (10:8.5:1.5)	2.997
n-Hexane–ethanol–water (10:8:2)	20.375
n-Hexane–ethyl acetate–ethanol–water (5:7:7:3)	1.652
n-Hexane–ethyl acetate–ethanol–water (5:5:7:3)	1.455
n-Hexane–ethyl acetate–ethanol–water (5:5:6.5:3.5)	2.313
n-Hexane–ethyl acetate–ethanol–water (5:5:6.5:3)	1.679

ethyl acetate–ethanol–water (5:5:6.5:3, v/v). In order to save solvents and time, the slow eluting compounds after the target substance were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was used only once. HPLC analysis of each peak fraction of this preparative HSCCC revealed that astaxanthin corresponding to peak A was over 97% pure. The HPLC chromatogram of astaxanthin as purified from the preparative HSCCC is shown in Fig. 3.

The *K* (partition coefficient) values of astaxanthin in several solvent systems were measured according to the literature [17], and are given in Table 1.

In conclusion, HPCCC was successfully used for the isolation and purification of astaxanthin from the microalga *Chlorococcum* sp. The present study indicates that HSCCC is a powerful tool in biotechnological down-stream processes for the separation and purification of biologically active substances.

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