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Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was successfully applied for the first time to the isolation and purification of the bioactive carotenoid zeaxanthin from the cyanobacterium *Microcystis aeruginosa*. The crude zeaxanthin was obtained by extraction with organic solvents after the microalgal sample had been saponified. Preparative high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v/v/v) was successfully performed yielding zeaxanthin at 96.2% purity from 150 mg of the crude extract in a one-step separation. The recovery of zeaxanthin was 91.4%. This was also the first report that zeaxanthin was successfully separated and purified from microalgae.

Keywords: *Microcystis aeruginosa*; Counter-current chromatography; Preparative chromatography; Zeaxanthin; Carotenoids

1. Introduction

The beneficial effects of carotenoids have been well documented from the numerous clinical and epidemiological studies in various populations. Carotenoids have been proposed as cancer prevention agents, life extenders, and the inhibitors of ulcer, heart attack and coronary artery disease [1–4]. Although a large number of dietary carotenoids can be absorbed into the blood circulation, only zeaxanthin is found present in human macular [5]. Recent studies have indicated that zeaxanthin may decrease the risk for age-related macular degeneration [6]. It is known that the concentration of the macular carotenoid can be manipulated by dietary intake of zeaxanthin. Zeaxanthin, however, exists only in a trace amount in common diet [7]. Certain algae may serve as a continuous and reliable source of zeaxanthin because they can be cultivated in bioreactors on a large scale. The chemical structure of zeaxanthin is shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography, which has been applied to the separation of a number of natural products [8–16]. However, no report has been published on the use of HSCCC for the isolation and purification of the highly bioactive zeaxanthin, in particular, from microalgae. The aim of the present study, therefore, was to develop an efficient method for the isolation and purification of zeaxanthin from the microalga *Microcystis aeruginosa* by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph

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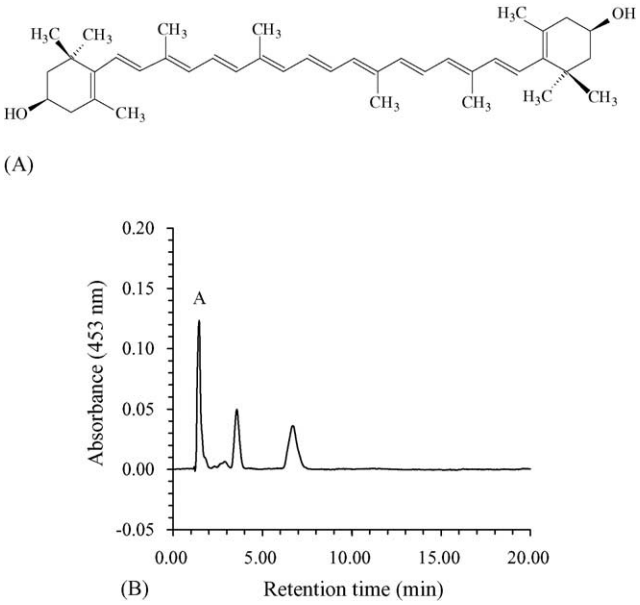

Fig. 1. (A) The chemical structure of zeaxanthin. (B) Chromatogram of crude zeaxanthin from the microalga *Microcystis aeruginosa* by HPLC analysis, A = zeaxanthin. Conditions: column: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase: methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v/v/v); flow-rate: 1.0 ml min^{-1}; detection at 453 nm.

(Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (inner 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.

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. Ethyl acetate, *n*-hexane, absolute ethanol, acetonitrile, dichloromethane and methanol were obtained from BDH (Poole, UK).

The microalga *Microcystis aeruginosa* was isolated from Lake Dianchi, Kunming, China, and cultured in our laboratory.

2.3. Preparation of crude zeaxanthin from Microcystis aeruginosa

Preparation of crude zeaxanthin was carried out according to Shi and Chen [17] and Li et al. [18]. In brief, 25 ml of 10.0 mol l^{-1} KOH solution-containing 2.5% ascorbic acid was added to 10 g of the dried alga, and the mixture was incubated at 60 °C for 10 min before cooling down to room temperature. A total of 10 ml of *n*-hexane–ethanol (1:1, v/v) was added to the mixture for the extraction of zeaxanthin. 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 evaporated to dryness by rotary vaporization at 40 °C. 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 solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system used for separation. A total of 10 ml of the sample solution-containing 150 mg of the crude zeaxanthin dissolved in *n*-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v) was injected.

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 in the head-to-tail elution mode 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 150 mg of the crude zeaxanthin was injected into the head of the column through the injection valve. The effluent of the column was continuously monitored with a UV–vis detector at 453 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 Shi and Chen [19]. The HPLC system used throughout this study consisted of two Waters 510 pumps (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 Millennium chromatography data system (Waters).

The column used was a reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm, Waters). The mobile phase was methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v/v/v) and the flow-rate was 1.0 ml min^{-1}. The effluent was monitored at 453 nm.

3. Results and discussion

The crude zeaxanthin obtained from *Microcystis aeruginosa* was analyzed by HPLC, and the chromatogram is shown in Fig. 1. Peak A corresponds to zeaxanthin. The purity of the crude zeaxanthin was 37.6%.

The selection of the two-phase solvent system is the most important, and is also the most difficult step because any change of the mobile phase composition is likely to change the stationary phase composition or volume; it is estimated that about 90% of the entire work in HSCCC has focused on this area. In order to select a suitable two-phase solvent system, some rules need be considered. For example, the target compound should be soluble and stable in the solvent system; the settling time of the solvent system should be short (<30 s); the partition coefficient (*K*) of the target compound should fall within a suitable range (usually between 0.5 and 2); and the retention of the stationary phase should be satisfactory [16,20–22]. Small *K* value usually results in poor resolution, while large *K* value tends to give better resolution but broader peak and more dilute peak fraction due to a longer elution time. The higher the retention of the stationary phase, usually the better the peak resolution.

Preliminary HSCCC studies were carried out with the two-phase solvent system composed of *n*-hexane–ethanol–water at a volume ratio of 4:3:1. The time to elute zeaxanthin was about 1.5 h, but zeaxanthin failed to separate from other compounds. As shown in Table 1, the *K* value of zeaxanthin in the two-phase solvent system was small (0.431). Thus, the two-phase solvent system composed of *n*-hexane–ethanol–water was not suitable for the separation of zeaxanthin from the microalgae. In the subsequent studies, another two-phase solvent system was thus tested.

Performance of the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios (5:5:6.5:3, 5:5:7:3 and 8:2:7:3) was evaluated in terms of peak resolution. This solvent system was more desirable than the other commonly used two-phase solvent systems that contain organic chloride (such as chloroform and tetrachloromethane) when environmental and health questions were considered. It required a long elution time (more than 5 h) to elute zeaxanthin with the two-phase solvent system (*n*-hexane–ethyl acetate–ethanol–water) at a ratio of 5:5:6.5:3, because the *K* value was relatively large (1.925). When the two-phase solvent system at a ratio of 5:5:7:3 was tested, the retention of the stationary phase was small, and it was very difficult to separate zeaxanthin from other components, although the elution time was shorter due to a smaller *K* value (1.782). When the two-phase solvent system at a ratio of 8:2:7:3 was used, the retention of the stationary phase was very high (68%), leading to satisfactory peak resolution. Fig. 2 shows the preparative HSCCC separation of 150 mg of crude sample using the solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v/v/v). After the zeaxanthin was eluted, in order to save solvents and time,

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

Solvent system	*K* value
n-Hexane–ethanol–water (4:3:1)	0.431
n-Hexane–ethyl acetate–ethanol–water (5:5:6.5:3)	1.925
n-Hexane–ethyl acetate–ethanol–water (5:5:7:3)	1.782
n-Hexane–ethyl acetate–ethanol–water (8:2:7:3)	1.384

Note: The *K* is the solute concentration in the upper phase divided by that in the lower phase.

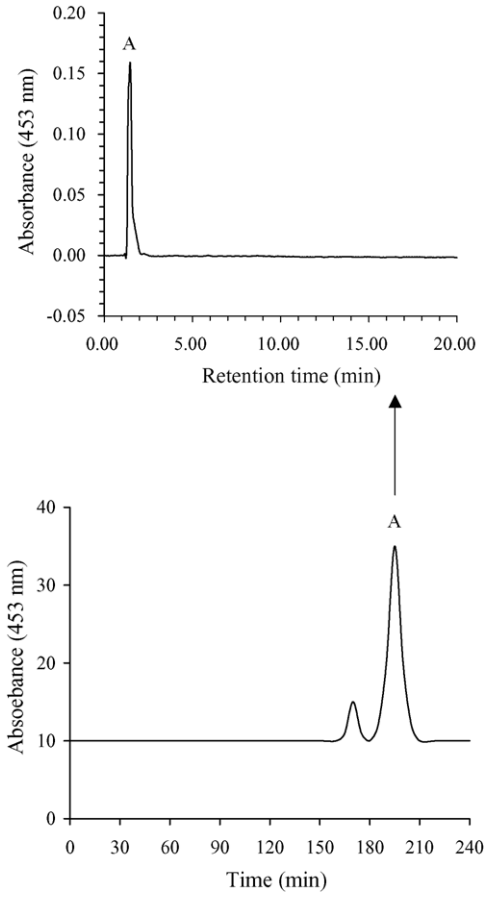

Fig. 2. Chromatogram of crude zeaxanthin from the microalga *Microcystis aeruginosa* by HSCCC separation, along with the HPLC chromatogram of purified zeaxanthin from HSCCC, A = zeaxanthin. HSCCC conditions: column: multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed: 1000 rpm; solvent system: *n*-hexane–ethyl acetate–ethanol–water (8:2:7:3, v/v/v/v); mobile phase: lower phase (water phase); flow-rate: 2 ml min^{-1}; detection: 453 nm; sample size: 150 mg; retention of the stationary phase: 68%. HPLC conditions: column: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase: methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v/v/v); flow-rate: 1.0 ml min^{-1}; detection at 453 nm.

the remaining compounds in the column were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was not to be reused. HPLC analysis of each peak fraction of this preparative HSCCC revealed that zeaxanthin corresponding to peak A was over 96.2% pure, and the recovery was 91.4%. The HPLC chromatogram of zeaxanthin as purified from the preparative HSCCC is shown in Fig. 2.

The *K* values of zeaxanthin in several solvent systems were measured according to the literature [22], and are given in Table 1.

In conclusion, HPCCC was successfully used for the first time for the isolation and purification of zeaxanthin from the microalga *Microcystis aeruginosa*, yielding zeaxanthin at 96.2% purity from 150 mg of the crude extract with the recovery of 91.4% in a one-step separation. This was also the first report that zeaxanthin was successfully separated and purified from microalgae. The present study indicates that HSCCC is a powerful tool for the separation and purification of biologically active substances in algae and has potential applications in other biotechnological downstream processes.

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