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NOTE

Simple and efficient isolation of cordycepin from culture broth of a *Cordyceps militaris* mutant

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Isolation of cordycepin from the culture broth of *Cordyceps militaris* mutant was investigated. Based on the solubility curve, three crystallizing processes, temperature shift (process I), pH shift (process II), and pH shift followed by temperature shift (process III) were carried out. Process III was the most promising method regarding both purity and yield.

[**Key words:** Cordycepin; *Cordyceps militaris* mutant; Proton beam irradiation; Crystallization; Purification]

Cordycepin is a nucleoside analog (3′-deoxyadenosine), which is the major active constituent of *Cordyceps militaris* and was first reported as a metabolite isolated from a culture broth of *C. militaris* (1). To date, cordycepin has been demonstrated to have many pharmacological activities, such as anti-tumor, anti-metastatic immunomodulatory and anti-inflammatory activities (2–5), and supply of a large amount of purified cordycepin is essential to the developments of its novel pharmacological use as well as its clinical trials. However, there are very few reports on commercially feasible procedures for isolation and purification of cordycepin. From the discovery of corycepin to the present, the purification methods have been based on column chromatography. Ion exchange chromatography using Dowex-I (Cl^-), Dowex-50 (NH_4^+) or Sephadex LH20 has been reported (6–8). The chromatography using Amberlite XAD-2 and silica gel followed by the two-step preparative HPLC was conducted by Ahn et al. (9). It is hard to apply the above methods to commercial scale purification due to the multi-step processes and the low recovery. In the latest reports, preparative HPLC (YMC-PAC C18) or macroporous DM 130 resin column chromatography were shown as a method for large scale preparations of cordycepin (10,11). However, even in these large scale methods, further steps such as butanol partition chromatography, silica gel column chromatography and recrystallization were necessary to get the final purified product.

Crystallization is a widely used separation process in a variety of industrial applications. Fortunately, the culture broth of the *C. militaris* mutant G81-3 obtained by the high-energy proton beam irradiation in our previous study, has high cordycepin concentration (12,13). Therefore, one step recovery of cordycepin from this culture broth by crystallization might be a promising method to produce purified cordycepin in bulk. In this study, some process factors for cordycepin crystallization from the culture broth of G81-3 were investigated to obtain a highly purified product with a high yield.

In order to produce cordycepin, the liquid surface culture of G81-3 was carried out as previously reported (13). The medium contained 86.2 g/L glucose, 93.8 g/L yeast extract (Difco) and Vogel's medium diluted to a 1/10 concentration. The culture was continued for 33 days at a temperature of 25 ± 1°C and a humidity of 30 ± 2% until the cordycepin production reached a plateau. The pH values of culture filtrate before and after the cultivation were 6.4 and 7.5, respectively. The concentration of cordycepin was determined by a Shimadzu HPLC apparatus (LC-10AT system) with a photodiode array detector (SPD-M10A) and a reverse phase column (TSK-gel ODS-80Ts, Tosoh Corp., Japan). The mobile phase consisted of methanol and 0.1% (v/v) phosphoric acid (2/98, v/v). The flow rate was 1.0 mL/min, and the column temperature was 40°C. The chromatogram was monitored by the absorbance range between 200 and 800 nm. The authentic standard of cordycepin was purchased from Sigma–Aldrich Co. As a result of the surface culture, more than 99% of the synthesized cordycepin was secreted to the liquid medium. Some products other than cordycepin (retention time, approximately 12 min) were detected as shown in Fig. 1A (no peak was detected between 400 and 800 nm). To evaluate the possibility of crystallization as a method of purification, cordycepin was tentatively crystallized from the culture filtrate as follows: (i) the freeze-dried filtrate of the culture broth (total cordycepin content approximately 15 g) was re-dissolved in distilled water at 70°C to produce a 20 g/L cordycepin solution; (ii) the solution was filtered through a membrane filter (pore size; 5-μm, Mixed cellulose ester A500A090C, Advantec, Japan) under reduced pressure for removal of insoluble components; (iii) the supernatant was left in

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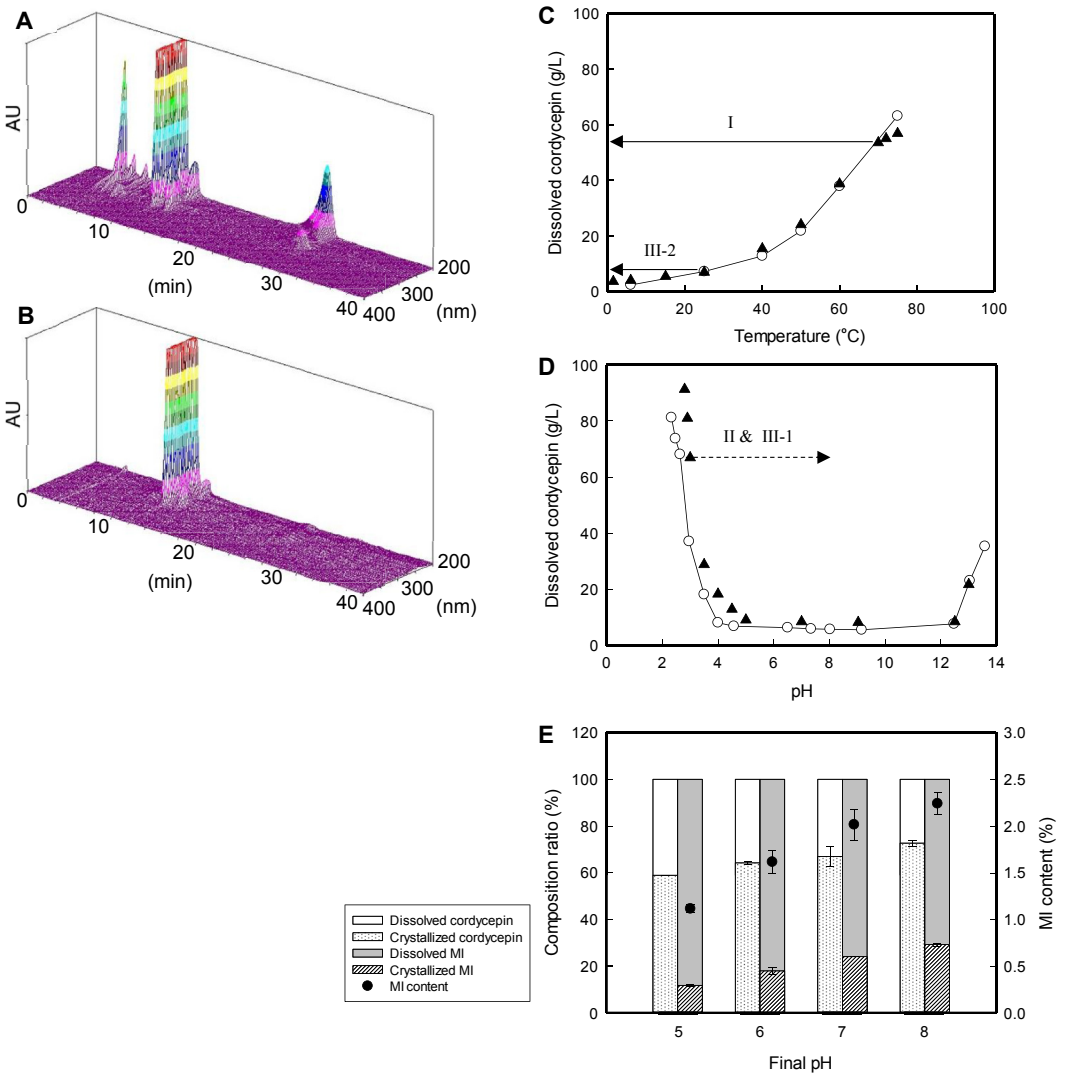

FIG. 1. (A) 3D HPLC profile of the culture broth of the mutant G81-3. (B) 3D HPLC profile of the tentatively purified cordycepin. (C) Solubility curve of cordycepin at different temperatures. pH was kept in the range of 6.4–6.5 (purified cordycepin), 7.1–7.2 (crude cordycepin). (D) Solubility curve of cordycepin at different pHs. Temperature was maintained at 25°C. Open circles and closed triangles show the results for purified and crude cordycepins, respectively. (E) Composition ratio of dissolved and crystallized compound for cordycepin and MI under the various final shifted pHs and MI content in the crystallized compounds (= cordycepin + MI) calculated from the former results.

the refrigerator (5°C) over night; (iv) the crystallized cordycepin was recovered with a filter paper (pore size, 7-μm); (v) the unwanted components like colored substances were removed by rinse with chilled distilled water; (vi) the crystallized cordycepin was freeze-dried. Fig. 1B shows the HPLC profile of the roughly-purified cordycepin as described above. The result indicates that the compound eluted just after cordycepin was the only main impurity, hereafter expressed as MI. The availability of crystallization as a method of purification has been demonstrated. The next target of the study was to obtain highly purified cordycepin with a high yield by decreasing the ratio of MI content.

The solubility of cordycepin was measured as follows under atmospheric pressure. Distilled water containing excess cordycepin had been stirred at different temperatures or pHs adjusted by a 5 mol/L of HCl solution and a 5 mol/L of NaOH solution until temperature or pH reached a set value and cordycepin concentration in the supernatant reached a constant value. At acidic region, the HCl solution and cordycepin were alternately supplied to stir smoothly and compensate for rise in pH by dissolution of cordycepin. The saturated concentration was defined as the solubility under the given condition. Two kinds of cordycepin powders with different purity were used to estimate the solubility. One was purified cordycepin obtained by the above tentative crystallization from the culture broth of G81-3. The other was crude one, that is, freeze-dried filtrate of culture broth itself. Fig. 1C and D shows the dependency of cordycepin solubility on temperature and pH, respectively. The solubility of cordycepin decreased with a decrease in temperature and dramatically increased at acidic and basic regions compared to the neutral region for both purified and crude cordycepins. The full fluctuation ranges of MI solubility by temperature and pH changes were less than those of cordycepin. According to the LC/MS analysis of the mixture of cordycepin and MI, the mass spectra of the LC peaks of cordycepin and MI gave the same main peaks at $m/z = 252.1$ corresponding to the protonated molecular ion $[M + H]^+$ peak of cordycepin. Furthermore, only MI was adsorbed on the cationic exchange resin. Therefore, the MI is presumed to be a cationic cordycepin analog. Detail investigations on structure and functions of MI are future subjects. On the basis of the results in Fig. 1C and D, three processes of cordycepin purification were designed so that the same recovery yield would be obtained. They were the crystallizations by lowering temperature shown as the arrow I in Fig. 1C (process I), changing pH from acidic to neutral shown as the arrow II in Fig. 1D (process II), and the combination of changing pH and lowering temperature shown as