Efficient purification of xanthomonasin A and B from *Monascus* yellow colorant by high-speed countercurrent chromatography

(Received July 7, 2010)  
(Accepted October 12, 2010)

Koichi Inoue a,1, Yuka Ito a,1, Yasuko Hattori b,1, Kaname Tsutsumiuchi c,1, Sumio Ito d,1, Tomoaki Hino a,1, b, Hisao Oka a,1

a) Department of Physical and Analytical Chemistry, School of Pharmacy, Kinjo Gakuin University  
b) Graduate School of Human Ecology, Human Ecology Major, Kinjo Gakuin University  
c) College of Bioscience and Biotechnology, Chubu University  
d) San-Ei Gen F.F.I. Inc.

Abstract

Recent studies reported that *Monascus*-fermented colorants have the biological effects such as anti-tumor initiation and anti-inflammation activity. In this study, we report the first procedure for the high, efficient, and low-cost purification of xanthomonasin A and B from *Monascus* yellow colorant. Purification involves high-speed countercurrent chromatography (HSCCC) with a simple two-phase solvent system composed of ethyl acetate/n-butanol/water (4/1/5, V/V). The HSCCC fractionated effluent peaks indicated that the peak resolution (Rs) was 1.8 between xanthomonasin A and B for 10 mg of loaded *Monascus* yellow pigment. These purified xanthomonasin A and B were evaluated by liquid chromatography tandem mass spectrometry with scan and daughter scan positive-modes, and the wide absorbance from 200 to 500 nm was monitored by photodiode array detection. The separation yielded 2.7 and 0.6 mg of xanthomonasin A and B (>95% purity) by HSCCC purification. These reference standards of xanthomonasin A and B can be used for various evaluations of their pharmacological activities. In addition, HSCCC purification would be useful to obtain main compounds from crude extracts.

Keywords: *Monascus* yellow colorant, xanthomonasin, high-speed counter-current chromatography, liquid chromatography-tandem mass spectrometry

I Introduction

*Monascus*-fermented rice has traditionally been used as a natural food colorant and food preservative of various foods in Asian countries. In addition, the biologically active components in the *Monascus*-fermented rice were identified by various analytical techniques. These components are well-accepted as a dietary supplements. Recently, various biological components in *Monascus*-fermented rice were referred in a review. Therefore, each component in *Monascus*-fermented rice has attracted attention for discovering new supplement and medicine.

*Monascus* yellow from *Monascus*-fermented rice have the inhibition of the mutagenicity and selective cytotoxic effects. Moreover, Lee *et al.* discussed that the certain composition of yellow pigment is identified as monascin, which has been shown as an anti-inflammation agent exhibiting potent inhibitory effects on 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. On the other hands, Watanabe *et al.* reported that the main yellow coloring compounds are analyzed and identified to xanthomonasin A and B by micellar electrokinetic chromatography. Therefore, we need to evaluate the *Monascus* yellow colorant and purify the main components for the many studies as reference standards in animal studies and analytical evaluations.

A number of studies using high-speed countercurrent chromatography (HSCCC) have suggested that very simple, efficient, and low-cost purification of natural products can be achieved. An HSCCC method for the purification of these main components from *Monascus* yellow colorant, however,
has not been reported. Here we performed simple and efficient purification of Monascus yellow components by HSCCC using a two-phase solvent system. The purified components were analyzed and identified by liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

II Materials and Methods

1. Materials and chemicals

Monascus yellow colorant was obtained from San-Ei Gen F.F.I. Co., Inc. (Osaka, Japan). HPLC-grade water, n-hexane, ethyl acetate, n-butanol, methanol, and formic acid (FA; 99%, LC/MS-grade), were obtained from Wako Chemical Co., Inc. Purified water was obtained using a Milli-Q Simplicity® UV system (Millipore, Bedford, MA, USA).

Concentrated solutions of Monascus yellow colorant were diluted as required by the addition of methanol/water (50/50, V/V).

2. HPLC analysis of Monascus yellow components

High-performance liquid chromatography (HPLC) was performed using a LC-20AD pump, SPD-20AV detector, CTO20AC column oven with injector and C-R8A recorder system (Shimadzu Co., Kyoto, Japan). TSK-GEL ODS-100V column was used (4.6 × 150 mm, 5.0 μm, Tosoh Co., Tokyo, Japan) for the separation of Monascus yellow components. The mobile phase for HPLC analysis consisted of 0.1% FA in water (Solvent A)/0.1% FA in methanol (Solvent B) with a flow rate of 1.0 mL/min. The column temperature was 40°C. A sample volume of 10 μL was injected. The HPLC gradient was as follows: 55% Solvent B at 0 min, 90% Solvent B at 15 min. The elution of Monascus yellow components was monitored by visible absorbance at 420 nm.

3. Determination of partition coefficients

One-mg of dried Monascus yellow colorant was added to two mutually equilibrated solvent phases (1 mL each; see Table 1) in a test tube, mixed to equilibrate, and then centrifuged (8000 rpm, 10 min). After settling, equal volumes of the upper and lower phases were transferred into separate test tubes, which were then evaporated and diluted using equal volumes of methanol/water. Each phase was assessed by HPLC, and the area of each peak was used to determine the partition coefficient (K) values for each component. The K value was calculated as follows:

$$K = \frac{\text{HPLC peak area of solute in upper phase}}{\text{HPLC peak area of solute in lower phase}}$$

4. HSCCC isolation of Monascus yellow components

High-speed counter-current chromatography (HSCCC) was performed using an HSCCC model CCC-1000 (multi-layer coil planetary centrifuge, Pharma-Tech Research Co., Baltimore MD, USA) with a 7.6 cm orbital radius that produces a synchronous type-J planetary motion with a maximum speed of 1000 rpm. This centrifuge was equipped with three column holders and three multilayer coiled columns. Each multilayer coiled column on the holder consists of nine coiled layers of 1.6 mm, polytetrafluoroethylene (PTFE) tubing with capacity of about 120 mL. All three columns are connected in series to provide a total capacity of about 350 mL. The beta values of the coil range from 0.5 at the internal terminal to 0.75 at the external terminal.

The two-phase solvent system composed of ethyl acetate/n-butanol/water (4/1/5, V/V) at room temperature was thoroughly equilibrated in a separatory funnel by repeated vigorous shaking three separate times, and followed by inverting the vessel and manipulating its stopcock. First, the column was entirely filled with the upper stationary phase. Secondly, 10 mg of dried Monascus yellow colorant was dissolved in 2 mL of each phase. Finally, these supernatant was then loaded into the column. The column was rotated at 756 rpm, while the lower mobile phase was pumped into

<table>
<thead>
<tr>
<th>Two-phase solvent system</th>
<th>Ratio (V/V)</th>
<th>Partition coefficient (K) ± SD (n=3)</th>
<th>Separation factor (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane/ethyl acetate/n-butanol/methanol/water</td>
<td>4/5/4/5</td>
<td>0.05 ± 0.01</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>3/5/3/5</td>
<td>0.13 ± 0.01</td>
<td>5.31</td>
</tr>
<tr>
<td></td>
<td>2/5/2/5</td>
<td>0.39 ± 0.02</td>
<td>3.42</td>
</tr>
<tr>
<td></td>
<td>1/5/1/5</td>
<td>0.43 ± 0.02</td>
<td>2.89</td>
</tr>
</tbody>
</table>
the head of the column at a flow-rate of 2.0 mL/min using an HPLC pump (LC-6A, Shimadzu Co., Kyoto, Japan). The effluent from the outlet of the column was fractionated into test tubes at 1 min/tube using a fraction collector (Model 2128 Fraction Collector, Bio-Rad Laboratories, Inc., NY, USA). The HSCCC fractions were analyzed by flow injection analysis (FIA). This analysis was performed using a LC-20AD pump, SPD-20AV detector, SIL-20AC auto-sampler, and C-R8A recorder system (Shimadzu Co., Kyoto, Japan). The solvent for FIA was water/methanol (50:50, V/V) with a flow rate of 1.0 mL/min. These HSCCC fractions were monitored at 420 nm.

5. Peak resolutions (Rs) of xanthomonasins A and B for HSCCC

Based on HSCCC fraction curve of xanthomonasins A and B, we calculated the peak resolutions (Rs) as follows:

\[ Rs = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5t1} + W_{0.5t2}} \]

The retention time and peak width of the midpoint for each compounds are t and W0.5ts, respectively.

6. LC/MS/MS analysis of xanthomonasins A and B from HSCCC purification

LC analyses were performed using a Waters Alliance 2695 system (Waters, Milford, MA, USA). LC separation was performed using a TSK-GEL ODS-100V (2.0 × 150 mm, 3 μm; Tosoh Co., Tokyo, Japan) maintained at 40°C. The mobile phase consisted of 0.1% FA in water (Solvent A) and 0.1% FA in methanol (Solvent B). The LC gradient was as follows: 55% Solvent B at 0 min, 90% Solvent B at 15 min with a flow rate of 0.2 mL/min. The injection volume was 2 μL. The mass spectrometer (a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA, USA)) was operated with an electrospray ionization (ESI) source in the positive mode. The ionization source conditions were as follows: capillary voltage of 3.0 kV, extractor voltage of 4 V, RF lens voltage of 0 V, source temperature of 110°C and desolvation temperature of 400°C. The cone and desolvation gas flows were 50 L/hr and 850 L/hr, respectively, and were obtained using a nitrogen source (N2 Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). Argon was used as the collision gas and was regulated at 0.35 mL/hr, setting the multipliers to 650 V. MS and daughter scan ranges were adjusted from m/z 50 to 700, and m/z 50 to 450. Cone voltage and collision energy were selected to 25 V and 15 eV, respectively. For the evaluation of purified compounds, the detection of 250 nm was used by LC with photodiode array detection (200 to 500 nm).

7. Nuclear magnetic resonance (NMR) analysis of xanthomonasins A and B from HSCCC purification

1H-NMR spectra were recorded on a JEOL AL-400 spectrometer (JEOL, Tokyo, Japan). Samples were studied as solutions in CD3OD (Cambridge Isotope Laboratories, MA, USA) at room temperature. Tetramethylsilane was used as an internal standard. Total assignments of the proton signals were made through COSY experiments.

### III Results and Discussion

1. Partition coefficients (K) of two components from Monascus yellow colorants

For achieving successful HSCCC separation to obtain pure standards of the main components, the two-phase solvent system should satisfy the three requirements for settling time, partition coefficient (K), and separation factor (α). The settling time of the two-phase solvent system with samples should be less than 30 s, the partition coefficient (K) value of the analytes should be close to 1.0, the separation factor (α = Kw/Ks, Kw > Ks) should be greater than 1.5, the two-phase solvents should be nearly equal volumes for each phase, and the two-phase solvents should be a volatile solvent system. In this study, we used the simple two-solvent system of hexane/ethyl acetate/n-butanol/methanol/water. Thus, the analytical methods should be needed to monitoring these analytes for the determination of K values. For the chromatographic separation of yellow components of Monascus yellow colorant, HPLC with C18-based column was used because these components have similar structures. When TSK-GEL ODS-100V and a simple mobile phase of 0.1% FA in water/methanol were used, two peaks were separated and analyzed by 420 nm monitoring (Figure 1). Therefore, we decided to monitor these components with this system, and to measure the partition.

![Fig. 1. HPLC chromatogram of Monascus yellow components](image-url)

Component A: xanthomonasins A, Component B: xanthomonasins B

Columns are TSK-GEL ODS 100V (4.6 × 150 mm, 5.0 μm, Tosoh Co., Tokyo, Japan). Mobile phase is consisted of 0.1% FA in water/0.1% FA in methanol. Monitoring absorbance is visible 420 nm.
coefficient \((K)\) of the peaks using HPLC chromatograms to select a two-phase solvent system for the HSCCC. The \(K\) and \(\alpha\) values of two \textit{Monascus} yellow components were shown in Table 1. These \(K\) values of two components for the solvent systems of hexane/ethyl acetate/\(n\)-butanol/methanol/water ranged from 0 to 13.5. By evaluating \(K\) and \(\alpha\) values shown in Table 1, it was determined that the two-phase solvent system of ethyl acetate/\(n\)-butanol/water (4/1/5, \(V/V\)) could be used to separate these \textit{Monascus} yellow components. This solvent composition provides satisfactory reproducible \(K\) values. In addition, Table 1 shows that the \(\alpha\) values of two components are greater than 1.5. This result proved that the ratio of 4/1/5 gives better settling time (<30 s) than other conditions. Thus, we decided to use this two-phase solvent system for the separation of two \textit{Monascus} yellow components by HSCCC.

2. HSCCC separation of \textit{Monascus} yellow components

This advanced HSCCC method can be useful for obtaining pure standards on the industrial scale.\(^{10, 11}\) Therefore, we have developed an efficient and effective HSCCC method for the purification of two components from \textit{Monascus} yellow colorant using the two-phase solvent system of ethyl acetate/\(n\)-butanol/water (4/1/5, \(V/V\)). We used 10 mg of dried \textit{Monascus} yellow colorant in the above-mentioned HSCCC system. Figure 2 shows the HSCCC elution curve of \textit{Monascus} yellow components, monitored at 420 nm by FIA. The retention of the stationary phase was 28%. The total separation time was 3 h, and the total elution volume was 360 mL. The HSCCC fractionated effluent peaks of A and B (Rs value: 1.8) was detected by FIA. Based on this elution curve, the collected fractions were combined into two pooled fractions of A and B (Figure 2). The amounts of the peak fractions were 2.7 (Fraction A) and 0.6 mg (Fraction B). The very low amount of component B remained in the column contents. However, we think that the Fraction B is enough for the separation of the high purity of component B by the HSCCC purification. These results showed that very efficient purification method for two \textit{Monascus} yellow components was developed by HSCCC using the simple two-phase solvent system.

3. Identification of xanthomonasain A and B by LC-MS/MS and NMR

An liquid chromatography (LC) with photodiode array (PDA) and mass spectrometry (MS) detectors was useful to identify the \textit{Monascus purpureus}-fermented rice.\(^{12}\) Moreover, LC-MS/MS can provide sufficient information to identify several components from \textit{Monascus} yellow colorant based on the review, tentatively.\(^{1}\) Based on the developed HPLC method for measuring \textit{Monascus} yellow colorant, we applied LC-MS/MS analysis using the column of smaller diameter for a flow rate of 0.2 mL/min than HPLC method. LC chromatograms of HSCCC fractions were shown in Figure 3. The photodiode-array (200–500 nm) chromatograms showed that the purity of the HSCCC fraction A and B were greater than 95%. For the calculations of purity, we used the HPLC chromatogram with 250 nm. The detections with 250 nm have many peaks of these fractions based on photodiode-array (200–500 nm) chromatograms. In addition, Figure 3 shows the LC with positive-MS scan and MS/MS daughter spectra of HSCCC fraction A and B. From the LC-MS/MS data of these HSCCC fraction A and B, we detected the \([M+H]^+\) ions (\(m/z\ 389\) for Fraction A, and \(m/z\ 417\) for Fraction B) of xanthomonasain A and B from other reports.\(^{1}\) As shown in MS/MS daughter scan mode (see Figure 3), these product ions of xanthomonasain A and B are corrected from the base peaks in the MS/MS spectra of protonated ion \([M+H]^+\). We also recorded theoretical fragmentation behavior of xanthomonasain A and B by quadrupole MS/MS with positive ESI mode. The \(^1\)H-NMR spectra of xanthomonasain A and B from HSCCC.

![Fig. 2. Elution curve and fraction peaks (Fraction A and B) of Monascus yellow colorant by HSCCC](image-url)
fractions were shown in Figure 4, respectively. Both spectra showed a singlet signal (9.46 ppm, 1H) due to the aldehyde group, a set of the trans olefin (5.72 ppm, dd, J_{HC} = 15 Hz, J_{HH} = 7 Hz, 1H; 5.41 ppm, dd, J_{HC} = 15 Hz, J_{HH} = 2 Hz, 1H), a doublet signal with geminal coupling constant J_{HH} = 18 Hz due to a methylene proton of a 6-membered ring (3.44 ppm, 1H), a multiplet signal due to another geminal proton and methylene protons adjacent to carbonyl group (2.92 ppm, 3H), and a multiplet signal due to two methyl groups on the olefin and the 6-membered ring (1.56-1.60 ppm, 7H). Methyl protons of the alkyl chain appeared as a triplet signal at 0.90 and 0.88 ppm (J_{HH} = 7 Hz, 3H) in the spectra of fraction A and B, respectively. The difference between the two similar spectra was only a multiplet methylene signal (1.28-1.34 ppm) of the alkyl chain. The intensities were 4H and 8H in the spectra of fraction A and B, respectively. Consequently, we could confirm the molecular structure of xanthomonasin A and B.

IV Conclusions

In this paper, we described the development of the high-quality purification of two Monascus yellow components (purity >95%) by HSCCC with a simple two-phase solvent system comprised of ethyl acetate/n-butanol/water (4/1/5, V/V). We evaluated the behaviors of the subsequent fragments of purified xanthomonasin A and B by quadrupole LC-MS/MS with positive ESI and MNR. It is suggested that the main components of Monascus yellow colorant are two xanthomonasin A and B, and that high purification of xanthomonasin A and B is very efficient and cost-effective. In addition, this advanced HSCCC approach would be easy to apply on the industrial scale for future works. These high-purity xanthomonasin A and B can be used in various studies involving biological and physical assays.

Fig. 3. LC/PDA chromatograms and MS/MS spectra of HSCCC purified xanthomonasin A (Fraction A) and xanthomonasin B (Fraction B)
Photodiode-array (PDA) detector is monitored from 200 to 500 nm. Total ion chromatograms (TIC) from m/z 50 to 750 are obtained by Waters Micromass Quattro Premier triple quadrupole mass spectrometer based on ESI-positive mode.
(1) HPLC analysis of fraction A from HSCCC by PDA (420 nm), (2) HPLC analysis of fraction A from HSCCC by PDA (250 nm), (3) HPLC analysis of fraction B from HSCCC by PDA (420 nm), (4) HPLC analysis of fraction B from HSCCC by PDA (250 nm), (5) MS spectrum of fraction A from HSCCC by ESI-positive mode, (6) MS/MS spectrum of fraction A from HSCCC by ESI-positive mode (m/z 389 →), (7) MS spectrum of fraction B from HSCCC by ESI-positive mode, (8) MS/MS spectrum of fraction A from HSCCC by ESI-positive mode (m/z 417 →)
Fig. 4. $^1$H-NMR spectra of HSCCC purified xanthomonasin A (Fraction A) and xanthomonasin B (Fraction B)

(1) Xanthomonasin A, (2) Xanthomonasin B; solvent, CD$_3$OD; temperature, 24°C; reference, tetramethylsilane; 400 MHz.

V Acknowledgment

This study was supported in part by The Japan Food Chemical Research Foundation (2008).

VI References


6) Watanabe, T., Yamamoto, A., Nagai, S., Terabe, S.; Separation and determination of Monascus...
論 文

高速向流クロマトグラフィーを用いたベニコウジ黄色素からの
キサントモナシン A 及び B の効率的な単離精製
(2010 年 7 月 7 日受付)
(2010 年 10 月 12 日受理)

井之上浩一 a)、伊藤有香 a)、服部晴子 b)、堤内 要 c)、伊藤澄夫 d)、日野知証 a,b)、岡 尚男 a,b)

a) 金城学院大学薬学部
b) 金城学院大学大学院人間生活研究科
c) 中部大学応用生物学部応用生物化学科
d) 三栄源エフ・エフ・アイ株式会社

キーワード: ベニコウジ黄色素、キサントモナシン、高速向流クロマトグラフィー、LC/MS

概 要

近年の研究において、ベニコウジ黄色素の各成分物質が抗ガン作用や抗炎症作用のようす生理活性を有することが注目されている。そこで、本研究では高速向流クロマトグラフィー (HSCCC) を用いたベニコウジ黄色素からのキサントモナシン A 及び B を効率的に単離精製する方法を開発した。HSCCC の二相溶媒には、酢酸エチル / プタノール / 水系 (4/1/5, V/V) を用いた。ベニコウジ黄色 10 mg を HSCCC で測定した結果、キサントモナシン A 及び B の分離度は 1.8 と良好に分離することができた。それらの単離精製された成分を液体クロマトグラフ / 質量分析法 (ESI- ポジティブモード) 及び紫外可視吸光光度法 (200-500 nm) により、分析評価を行った。その結果、純度 95% 以上 (HPLC) のキサントモナシン A (2.7 mg) 及び B (0.6 mg) を得ることができる。HSCCC で得られたキサントモナシン類は、様々な生理活性試験に応用できるものと思われる。更に、HSCCC は粗抽出物からの主成分の単離精製に有効な手段と考えられた。

連絡先: 〒463-8521 愛知県名古屋市守山区大森 2-1723 金城学院大学薬学部 井之上浩一