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High Yield Production of Anthraquinone by Cell Suspension Cultures of *Crocus sativus* L.

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We reported two kinds of cell-suspension cultures of saffron (*Crocus sativus* L.) One cell line is white, consisting of small cell aggregates and the other is yellow, consisting of large cell aggregates. The latter produce a yellow pigment that has an antimicrobial effect on some kinds of gram possitive bacteria. To analyse this pigment, it was isolated and identified, for the first time from cell-suspension cultures of saffron, as 3, 8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid.

Introduction

The main pigments of saffron are mono- and diglycosyl esters of polyene dicarboxylic acid crocetin. Minor amounts of β-carotene, γ-carotene, lycopene and zeaxanthin have also been isolated[1-3], and their production from stigma-like structures has been reported[4-6]. No reports are available on the production of anthraquinone from saffron or tissue cultures of saffron. We previously reported free amino acid production and high activity of phenylalanine ammonia lyase (PAL) in yellow and large cell aggregates[7].This paper describes the isolation of 3, 8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid for the first time from cell-suspension cultures of saffron. Variable amounts of pigment for different cell-suspension cultures of saffron, and antimicrobial effects on some kinds of microbes are also discussed.These results suggest that a higher production of this pigment may be possible from our cell-suspension cultures of saffron.

Materials and Methods

Induction of cell-suspension cultures. Cell-suspension cultures were obtained from saffron bulbs cultured on Murashige and Skoog[8] medium supplemented with 0.5 mg/*l* 2, 4-D and 0.3 mg/*l* Zeatin (MS medium) at 25°C in the dark as reported previously[7,9].

Isolation of yellow pigment and HPLC analysis. Lyophilized suspension cells (about 1 kg fresh weight), cultured in MS medium for 4 weeks, were mashed and extracted with MeOH 3 times. The extract was partitioned between water and EtOAc after being defatted 3 times by n-hexane to remove free fatty acids. The EtOAc phase after drying was subjected to silica gel (75-150 μm) column chromatography (EtOAc: chloroform: AcOH=6 : 4 : 2), and rechromatographed with an ODS-silica gel (200-300 μm) column chromatography (water: MeOH=3 : 7) to give the yellow pigment (40 mg). Samples extracted with EtOAc, after being dried with sodium sulfate anhydrous, were evaporated to dryness. Melting points were determined with a Melt-pointer (SHIBATA). The

samples thus obtained were dissolved in MeOH filtered by a membrane filter (0.45 μm) and injected (2-5 μl) into a HPLC column. The HPLC conditions were as follows: column; TSKgel ODS-80TM (7.8 mm i.d.×300 mm, TOSOH), mobile phase; water: MeOH=3:7, column temp.; 25°C, flow rate; 0.9 ml/min, detection; UV 254 nm.

Identification of isolated yellow pigment. ^{1}H and ^{13}C-NMR spectra were measured at 400 MHz and 100.57 MHz on a Varian VXR-4000S spectrometer. Mass-spectra were measured on a JEOL JMS-SX102.

Determination of antimicrobial activity. Antimicrobial activity was determined by the paper disc method[10]. Each paper disc contained 10 μg of test compounds (EtOAc extract). The following bacteria were used: *Staphylococcus aureus* (IFO 13276), *Escherichia coli* (ATCC 12041), *Zygosaccharomyces rouxii* (IFO 0680), *Lactobacillus planturum* (ATCC 14917). The following media were used: for the first 3 bacteria; YM medium (peptone 7.5 g, yeast ext. 3.0 g, malt ext. 5.0 g, glucose 10.0 g and H_2O 990 ml, pH 7.0), for the last bacteria; Lactobacilli MRS Broth (DIFCO LABORATORIES).

Results and Discussion

Induction of cell-suspension cultures.

In our previous work[7,9], large yellow cell aggregates and small white cell aggregates were obtained. The former produce yellow pigment which resist long time exposure to fluorescent light and strong acid (data not shown). It also has antimicrobial effects on some kinds of microbes (**Table 2**). This pigment was isolated and analyzed.

Isolation of yellow pigment and HPLC analysis.

The yellow cells produced about 7 times the amount of yellow pigment as the white cells, and saffron bulbs gave almost no pigment by our method (**Table 1**). Despite using the same medium, one cell line produced more yellow pigment than the other cell line. On some occasions, cultured plant tissues showed the above features and thus the effect of this pigment was considered selective[11,12]. Thus, a higher production of this pigment may be possible from our cell suspension cultures of saffron. The yellow cells produced more free amino acids than the white cells, and expressed higher activity of PAL[7]. Yellow cells may possibly have high activity for metabolism, and it follows that yellow pigment may be a second metabolite relative to PAL. Identification of this pigment was attempted on the basis of this consideration.

Identification of isolated yellow pigment.

^{1}H-NMR spectra were as follows; in DMSO-d_6/TMS: δ 12.95 (8-OH), 7.73 (1H, t, J=7.7/8.1 Hz, 6-H), 7.65 (1H, dd, J=7.5/1.3 Hz, 5-H), 7.56 (1H, s, 4-H), 7.34 (1H, dd, J=8.2/1.3 Hz, 7-H), 2.76 (CH_3, s). Correlations among 6-H, 5-H and 7-H were confirmed by ^{1}H-^{1}H COSY spectra. ^{13}C-NMR spectra were as follows; in CD_3OD/TMS: δ 20.5 (CH_3), 113.3 (4, CH), 118.4 (5, CH), 119.6 (2), 124.

Table 1. Amount of the pigment in the tissues.

	μg/g tissue (fresh weight)	relative amount
yellow-cell	113.0	6.8
white-cell	16.5	1
bulb	2.3	0.04

column; TSKgel ODS-80TM (7.8 mm i.d. ×300 mm, TOSOH), mobile phase; water: MeOH=3:7, flow rate; 0.9 ml/min, detection; UV 254 nm.

Table 2. Antimicrobial activity toward 4 kinds of microbes.

	yellow-cell	white-cell	bulb	purified pigment
Staphylococcus aureus IFO 13276	21.0	11.0	10.0	9.0
Escherichia coli ATCC 12041	—	—	—	—
Zygosaccharomyces rouxii IFO 0680	—	—	—	—
Lactobacillus planturum ATCC 14917	12.0	—	—	8.0

Each paper disc contained 10 μg of test compounds (Et0Ac ext.).
Presented by the inhibited zone of the microbes (diameter, mm).
— showed no effect toward the microbes

6(7, CH), 125.6, 132.3, 134.2(6, CH), 136.9, 138.5, 143.1(1), 160.3(3), 163.5(8), 171.1(COOH), 183.7(10), 191.1(9). Signal multiplicities were clarified by DEPT experiment. Correlations between ^{13}C and ^{1}H were confirmed by ^{13}C-^{1}H COSY spectra. EI-MS spectra were as follows: m/z(%)=298 (81) [M^+], 280(100) [M^+-H_2O], 252(13), 224(17), 196(6), HR-MS: M/Z=298.0476, calc. 298.0477, $C_{16}H_{10}O_6$. M. p. =244-248°C (lit[13]:248-250°C). Based on the above data, the pigment was identified as 3, 8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid. It has already been detected in cultures of Streptomycete[13] and ancient dyestuff[14] (CAS-Registry No.: 69119-31-9). Neverthless, this is the first report on its isolation from saffron tissue or its tissue cultures[1-6].

Determination of antimicrobial activity.

Table 2. showed the antimicrobial activity of 4 kinds of microbes. The purified pigment elpressed considerably strong activity, particularly toward *Staphylococcus aureus* and *Lactobacillus planturum*, both harmful microbes in foods. Among all substances examined, yellow cells had the strongest activity. The yellow cells produced yellow pigment in an amount 7 times that of white cells, and the activity of the purified pigment from the yellow cells was closer to that of yellow cells in the case of *Lactobacillus planturum* than *Staphylococcus aureus*, so we considered the purified pigment expressed to have stronger activity toward *Lactobacillus planturum*. Other unknown components from the tissues of yellow cells would surely have caused the high activity toward *Staphylococcus aureus*. These microbes are harmful in foods and consequently, the possibility of using anthraquinone pigment as a food-additive and its relationship to the metabolism of cell-suspension cultures of saffron are being studied.

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《和文要約》

サフラン培養細胞からのアントラキノン系色素の高生産

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サフランの造る色素はクロセチンを代表とした，カロチン系の色素であり，アントラキノン系色素が報告された例は，培養細胞を含めても今までなかった．我々はすでに，サフラン培養細胞における遊離アミノ酸の定量やPAL活性の測定を報告してきた．今回，既知ではあったがサフラン培養細胞では始めてアントラキノン系色素の生産を見出し，培養細胞間に生産性の差があることを明らかにするとともに，この色素のもつ抗菌活性などについて知見を得たので併せて報告する．