Kinobeon A, A Novel Red Pigment Produced in Safflower Tissue Culture Systems

Sachio Wakayama, Kazuhito Kusaka, Tsutomu Kanehira
Kibun Foods Inc., 7-14-13 Ginza, Chuo-ku, Tokyo 104

Yasuyuki Yamada
Faculty of Agriculture, Kyoto University, Kyoto 606

Kazuyoshi Kawazu, and Akio Kobayashi
Faculty of Agriculture, Okayama University, Okayama 700, Japan

Z. Naturforsch. **49 c**, 1–5 (1994); received October 12/November 11, 1993

Kinobeon A, Red Pigment, Safflower, *Carthamus tinctorius,* Plant Tissue Culture

The production of safflower pigments by tissue culture techniques was carried out using the calli induced from various parts of the plant. After massive cell selection efforts, a culture cell line (KB 7) was found to produce a considerable amount of a red pigment. Addition of cellulose powder and D-phenylalanine into the medium dramatically improved the pigment production. After purification, red crystals were obtained. Its UV/VIS spectrum as well as the HPLC behavior was clearly different from that of carthamin found in the mother plant and those of another typical plant pigments, suggesting that it was a novel compound. Therefore, this pigment was named kinobeon A.

Introduction

Safflower (*Carthamus tinctorius* L.) belonging to Compositae family is a biennial plant [1]. The red pigment complexes obtained from the processed yellow flowers have been traditionally used as a dyestuff and the dried material has also been used as a folk medicine for women's disease. The production of secondary metabolites by plant tissue or cell culture has attracted our special interest since these compounds are useful for medicines, food additives and cosmetics. Many papers have been reported about production of secondary metabolites by plant cell culture methods [2–4]. In our laboratory much attention has been paid to the production of safflower pigments. So, we tried to induce the safflower callus and to establish the pigment-producing cell culture system. In proceeding isolation, the chromatographic and spectroscopic behaviors of the red pigment were found to be clearly different from those of typical plant pigments. This paper deals with the production of a new pigment, kinobeon A and its isolation together with its spectroscopic features.

Reprint requests to Prof. Dr. A. Kobayashi.

Verlag der Zeitschrift für Naturforschung,
D-72072 Tübingen
0939–5075/93/0100–0001 $ 01.30/0

Materials and Methods

Chemicals

Carthamin, delphinidin-3-rutinoside and betanin were kindly supplied by Dr. K. Saito (Hokkaido Tokai University, Sapporo). Cellulose powder, phenylalanine (D-, L-form), tyrosine (D-, L-form), and tryptophan (D-, L-form) were obtained from Wako Pure Chemical (Osaka). Cinnamic acid and sinapic acid were purchased from Tokyo Chemical Industry (Tokyo) and β-carotene was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Other chemicals and reagents were all analytical grade and commercially available.

Plant material

Safflower seeds were donated by a farmer, Mr. H. Ohgawara in Yamagata prefecture. Two months after sowing, young leaves excised from the upper part of the plant were soaked in 0.5% Tween 20 solution containing 2% sodium hypochlorite for 5 min, and then rinsed three times with sterile water. The leaves were cut into small sections (*ca.* 5 × 5 mm), which were placed on the KG medium (modified MS medium [5]) listed in Table I. The callus formation started 5 days after the leaf section was placed on the induction medium (KG medium, NAA: 10^{-6} M, BA: 10^{-6} M, gellan gum: 0.2%). The callus clumps induced sporadically from

the leaf sections were dissected and transferred onto the fresh KG medium (gellan gum: 0.2%). Repeated transplantation to a new medium resulted in marked growth enhancement (increase in weight by two times in 3 days).

Cell selection

The callus clumps were smashed into small aggregates by passing through the nylon mesh (300 μm). After 2-week incubation several cell clumps with high proliferation rate were selected as host cells, on which a filter paper moistened with the MS medium was placed, and then small clamps were set (Fig. 1). Random combination of host cells (H 5, 8, 11) with high proliferation rate and the aggregates (R 8, 15, 16, 20) with low growth rate was provided and the filter paper sandwiched by host cells and an aggregate was found to be pinkish. The aggregates were transferred into 50 ml of the liquid KG medium to establish suspension culture. The growth of the two cell lines, R 8, 16 was improved in the suspension culture and the high proliferation rate was successively maintained for at least 5 months. The suspension cultures were employed to select the most promising cell lines with high productivity of kinobeon A.

Pigment production

At the fourth day after being transferred to the new medium, the cells were collected with nylon mesh (100 μm) and *ca.* 3.5 g of the filtered cells was transferred to 75 ml of the fresh KG medium containing 3 g of cellulose powder. After 4-day shaking, the cultured cells and the pinkish powder were collected by filtering the medium and dried at 4 °C in the dark. The filtered cake was extracted with methanol, and the fixed amount of the methanolic concentrate was spotted on Kieselgel 60 F_{254} plate (Merck, Darmstadt, Germany), which was developed in benzene/acetone/methanol (7:2:1, v/v),

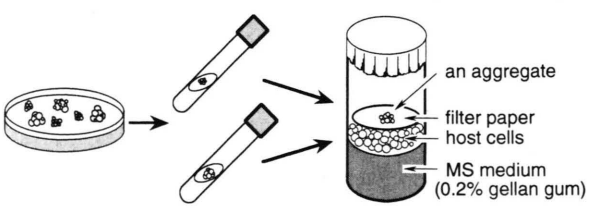

Fig. 1. Cell selection and nurse culture.

and the pigment content was measured at 520 nm with a Shimadzu Dual Wavelength Flying-Spot Scanner CS-9000. A cell line (KB 7) was selected as the most potent cell line to produce a red pigment.

Pigment production medium

After a 4-day suspension culture the KB 7 cells were employed to determine the optimal condition for the pigment production. A spoonful of the precultured cells (*ca.* 3.5 g) was placed in a 300 ml flask containing 3 g of cellulose powder/75 ml of the KG medium, and then cultured on the rotary shaker in darkness (75 rpm, 25 °C). Several inorganic constituents of the KG medium were removed in turn, and 1 mM of several plausible flavonoid precursors such as phenylalanine, tyrosine, tryptophan, cinnamic acid and sinapic acid were added to the KG medium containing cellulose powder. After 4-day shaking, the medium was filtered with sintered glass and the resulting pinkish powder was dried at 4 °C in the dark. One gram of the powder was soaked in 5 ml of pyridine and left to stand for 5 min. Absorbance of the supernatant was recorded at 520 nm with a Shimadzu UV-265.

Purification of the red pigment

The filtered cake (950 g) was dried at 4 °C in the dark and soaked in 3.2 l of acetone/methanol (1:1, v/v) at room temperature for 2 h. The extract was filtered through sintered glass. The filtrate was condensed *in vacuo,* and then passed through a Toyopearl HW-40 column (2.5 × 50 cm) using acetone/methanol (1:1, v/v) as an eluant. The red color fractions were combined, concentrated to give a syrup, which was chromatographed on Sephadex LH-20 (1.6 × 60 cm) using methanol as an eluant. The resulting fractions were combined and concentrated to give a red residue. Recrystallization was carried out at room temperature using methanol.

Results

Establishment of a pigment-producing medium

It is known that a variety of stresses act as stimuli toward secondary metabolite production in tissue culture systems. In this experiment, addition of cellulose powder (3 g/75 ml of KP medium) was found to be essential to the kinobeon A production.

Several plausible flavonoid precursors such as phenylalanine, tyrosine, tryptophan, cinnamic acid and sinapic acid were also added to the modified KG medium. As shown in Fig. 2, addition of D-phenylalanine (1 mM) and D-tyrosine (1 mM) markedly increased pigment productivity about 14.3 and 3.5 times, respectively. However, L-isomers as well as other plausible precursors were not effective. The effects of metal ion deficiency on the pigment formation were examined. Elimination of Ca^{2+} and Mg^{2+} from the medium was found to be promising (Fig. 2). Based on these results, the pigment-producing medium, the KP medium, was finally established (Table I).

Selection of cell lines with high productivity of red pigments

By means of a plating method using the KG medium, cell lines with high pigment productivity were sorted. Four cell lines (R 8, 15, 16, 20) were selected as the nursed calli which accumulated the pigment on the filter paper, and after several selections R 8 and 16 were found to produce the pigment 1.5–2.1 times as much as the original cell lines. Fifty cell lines were established from the two suspension cultures (Rs 8, 16) possessing the highest pigment content. One cell line (KB 1) was finally sorted as the most promising line, and at the seventh selection from KB 1 the pigment content of one cell line (KB 7) reached 11.39 times as high as that of the original cell line. In order to improve the pigment production further, a two-step culture method was employed. KB 7 cell line was pre-cultured in the KG medium and then transferred into the KP medium. As shown in Fig. 3, the density of pinkish color of the cellulose powder gradually intensified with prolonged incubation period although KB 7 cell line did not propagate in this medium. This indicated that the two-step culture method was effective for kinobeon A production. Its productivity reached the maximum at the 4th day after the pre-cultured cells were transferred into the KP medium.

Table I. Growth and pigment production media for *C. tinctorius* cell culture.

Material[a]	Conc. [mg/l]
NH_4NO_3	825
KNO_3	950
KH_2PO_4	85
$CaCl_2 \times 2H_2O$[b]	0 [220]
$MgSO_4 \times 7H_2O$[b]	0 [185]
$MnSO_4 \times 4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4 \times 7H_2O$	8.6
KI	0.83
$NaMoO_4 \times 2H_2O$	0.25
$CuSO_4 \times 5H_2O$	0.025
$CoCl_2 \times 6H_2O$	0.025
$FeSO_4 \times 7H_2O$	27.8
Na_2EDTA	37.3
myo-Inositol	100
Thiamine hydrochloride	0.1
Pyridoxine hydrochloride	0.5
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30000
α-Naphthaleneacetic acid	0.186
N-6-Benzyladenine	0.225
D-Phenylalanine[b]	165.19 [0]
Cellulose powder[b]	40000 [0]

[a] 0.2% gellan gum was used in the solid KG medium.
[b] Contents were changed to [ ] in the KG medium.

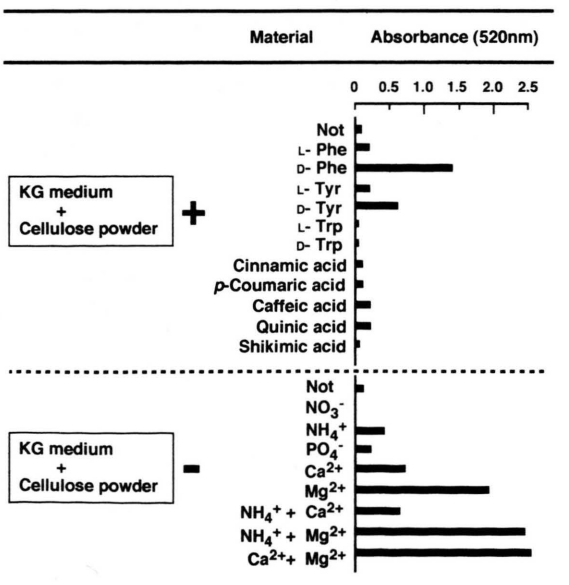

Fig. 2. Effects of various factors on pigment production.

Purification of the red pigment

Sephadex LH-20 column provided successful separation as shown in Fig. 4. The red pigment was eluted into the fractions, Nos. 15–19, which showed the R_f 0.44 on Kieselgel 60 F_{254} plate using benzene/acetone/methanol (7:2:1, v/v) as a developing solvent. The fractions, Nos. 20–23 and Nos. 24–28

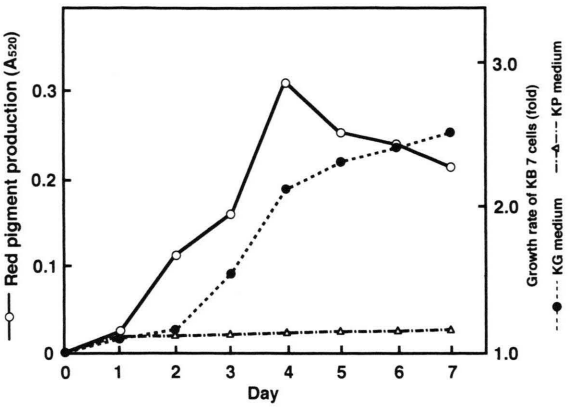

Fig. 3. Time course of pigment production and growth rate of KB 7 cells in KP medium and KG medium.

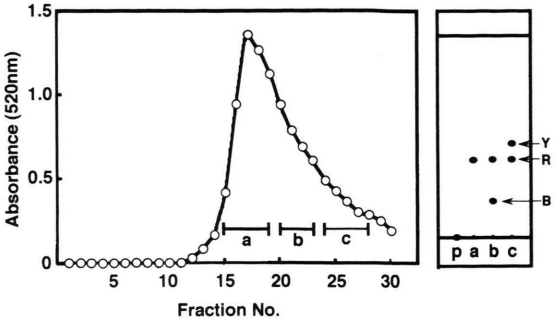

Fig. 4. Separation of the pigments by Sephadex LH-20 column eluted with methanol (left). TLC analysis of the fractions (right). p: carthamin (R_f 0), R: red spot (R_f 0.39), Y: yellow spot (R_f 0.46), B: blue spot (R_f 0.19).

were separately combined and developed on TLC. A blue component from the former fractions and a yellow component from the latter exhibited R_f values, 0.49 and 0.58, respectively. These two components were rather unstable, and further purification was not carried out. Carthamin, a red pigment from safflower (*Carthamus tinctorius* L.) retained at the original spotted position (Fig. 4). Chromatoscanning traces of these three components showed similar spectral features, suggesting that these components had a similar chromophore but different length of extended conjugation systems (Fig. 5). The fractions, Nos. 15–19 were combined and concentrated to give a red residue and the subsequent recrystallization from methanol gave red rods (2 mg). The melting point of this pigment was 224–226 °C. None of the melting points, so far reported in the literatures, were identical to that of this pigment. As shown in Fig. 6, this red pigment was considered to be novel since its UV/VIS spectral features [λ_{max} (MeOH) nm (ε): 520 (63,500)] were also different from typical red pigments from plants.

Discussion

In tissue culture systems, the proliferation rate is not proportional to the ability of secondary metabolite production [6, 7]. Pigment accumulation was not seen in the optimum growth medium (KG medium). However, addition of cellulose powder substantially resulted in success in the pigment formation. This raised a question that an eliciting factor originated from cellulose powder could stimulate secondary metabolite production. Oligosaccharides obtained by enzymic hydrolysis of naturally occurring polysaccharides or by autoclaving fungal cell walls were found to act as elicitors [8, 9]. Therefore, the supernatant from the long-term-autoclaved cellulose powder (40 min) and the acid-hydrolyzed fraction from the cellulose were added to the medium, but no positive improvement of the pigment production was seen (the data are not shown). The effects of cellulose powder on the pigment deposition was not yet clarified, but specific affinity of the pigment toward cellulose powder could slide the equilibrium of biosynthetic reactions into the products. Additionally, D-phenylalanine and D-tyrosine had a synergistic effect to improve pigment accumulation. Besides,

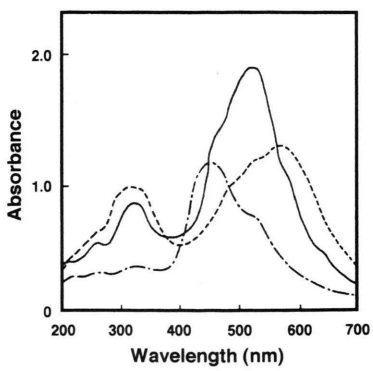

Fig. 5. Chromatoscanning traces of red, yellow and blue pigments separated by Sephadex LH-20 column. ——: Red component (kinobeon A); — - —: yellow component; – – – – –: blue component.

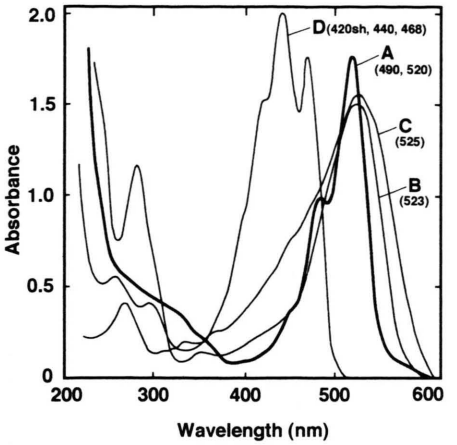

Fig. 6. UV/VIS spectra of typical plant pigments. A: kinobeon A, B: delphinidin-3-rutinoside, C: betanin, D: α-carotene (measured in the methanol solution).

elimination of major inorganic elements such as Ca^{2+} and Mg^{2+} also worked positively for pigment formation. Such nutrient modification is not favorable to cell growth, and the cells in the modified medium (KP medium) are therefore being cultured under severe stress conditions. The percentage of living cells 4 days after transferring to the KP medium was lower than that in the KG medium, which might suggest that some factors derived from damaged cells or the cell walls of dead cells could act as elicitors in the secondary metabolite production. Further investigation on this problem is in progress.

The red pigment was obtained as red pure crystals and showed sharp m.p. (224–226 °C). Its UV spectrum as well as the chromatographic behavior on TLC was not identical to those of natural pigments such as carthamin found in the mother plant, anthocyanidin, quinones, and carotenoids. None of the compounds so far reported exhibited identical physicochemical properties of this pigment. Therefore, this pigment was novel and named kinobeon A.

The formation of blue and yellow pigments were also determined in the KP medium together with kinobeon A. The spectral features given by a chromatoscanner suggested that these two pigments possessed basically the same chromophores as kinobeon A. Identification of these pigments in the flower extract of the mother plant (*Carthamus tinctorius* L.) was performed, but no colored spots corresponding to these red, blue and yellow pigments were observed. Now, identification of these pigments has been started in various parts of the plant at the different growth stages. The role of the amino acids to improve pigment formation is not clear. These D-amino acids might act as metabolic inhibitors, which block the biosynthetic pathways for other metabolites or further modification of the products, and consequently a considerable amount of secondary metabolites was produced in the suspension culture. Although the physiological action of these amino acids as well as cellulose powder are unclear, coincidental combination of the two factors, cellulose powder and D-phenylalanine brought us a break-through to the long-term struggle for the pigment production. Structural elucidation of kinobeon A is now in progress [10], and the result will be soon reported in the following paper.

Acknowledgement

This work was supported by a Grant-in-Aid to A. K. for Scientific Research (No. 02261104, No. 04252104) from the Ministry of Education, Science and Culture of Japan.

[1] T. Makino, Makino's New Illustrated Flora of Japan, p. 813 (1988).
[2] Y. Fujita, Y. Hara, C. Suga, and T. Morimoto, Plant Cell Rep. **1,** 61–63 (1981).
[3] M. Sauerwein, K. Yoshimatsu, and K. Shimomura, Plant Tissue Cult. Lett. **9,** 1–9 (1992).
[4] K. Ishimaru, M. Satake, and K. Shimomura, Plant Tissue Cult. Lett. **7,** 159–163 (1990).
[5] T. Murashige and F. Skoog, Physiol. Plant. **15,** 473–497 (1962).
[6] P. Das. Nagaratman and Y. Yamada, Annu. Reports ICBiotech. **10,** 409–411 (1987).
[7] M. J. C. Rhodes, R. J. Robins, A. J. Parr, and J. Hamill, J. Appl. Bacteriol. **63,** 105–114 (1987).
[8] A. Kobayashi, A. Tai, H. Kanzaki, and K. Kawazu, Z. Naturforsch. **48 c,** 575–579 (1993).
[9] C. A. Ryan, Biochemistry **27,** 8879–8883 (1988).
[10] S. Wakayama, K. Kusaka, T. Kanehira, Y. Yamada, K. Kawazu, and A. Kobayashi, Abstracts, The 15th International Botanical Congress, Yokohama, Japan, Aug. 28–Sept. 3, p. 368 (1993).