Accumulation of a Novel Red Pigment in Cell Suspension Cultures of Floral Meristem Tissues from *Carthamus tinctorius* L.

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A cell strain originally derived from floral meristem tissues of *Carthamus tinctorius* (dyer's saffron) produced substantial amounts of a novel red pigment under controlled culture conditions. The pigment isolated from alcoholic extracts of *C. tinctorius* cultures was compared with authentic carthamin, anthocyanins, betain, and carotenoids. It differed markedly from carthamin and showed none of the characteristic properties of the glycoside or chloride forms of authentic delphinidin, cyanidin, and pelargonidin. Analytic data indicated that this pigment also differs from betanin and from α- and β-carotene. The name "Kurenamin" was tentatively given to this newly isolated red pigment. Effect of the culture media, micro-elements, macro-elements, and putative substrates on the kurenamin production were investigated during cell suspension culture.

Introduction

Cell suspension cultures from certain plant tissues provide suitable systems for the study of the production and accumulation of natural colouring matters [1–4], because the cultured cells have the advantage over intact plants of being freely obtainable when required and in frequently having high pigment productivity. Moreover, they can be studied under strictly controlled conditions.

Intact flowers of *C. tinctorius* (dyer's saffron) produce carthamin, a traditional red dye used to colour rouge and cotton textiles [5]. This pigment accumulates in the upper half of the tubular *Carthamus* flowers at the colour transition stage, usually just before floret wilting [6]. The localized and specified occurrence of this colouring matter suggests that carthamin synthesis is induced under a particular condition that is closely related to the physiological status of the matured tissues of *C. tinctorius* flowers. As yet, there is no evidence that indicates the initiating factors and/or the controlling mechanism of pigment formation in the intact flowers or in the cultured tissues of this plant. During our study of variations in phenol-oxidizing enzyme activities in the callus cultures from the epicotyl tissues of *C. tinctorius* seedlings, we found that the activity of a carthamin-synthesizing enzyme was always 10- to 35-fold that in the parent plant, even though no carthamin was found in itself, was detected in our cultures [7]. We, therefore, established a cell culture in which synchronous pigment synthesis occurred under defined culture conditions, and here report the first evidence of a red pigment accumulation in *C. tinctorius* cultures. We also give a partial characterization of the pigment and describe the effect of culture conditions on its production in habituated cell cultures from the floral meristems of the capitula of *C. tinctorius*.

Materials and Methods

Chemicals

The chemicals used were from the following sources: carthamin was from our laboratory collec-

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Abbreviations: LS, Linsmaier-Skoog; MS, Murashige-Skoog; KBC-G, Kibun basic culture-growth; KBC-P, Kibun basic culture-pigment production; BA, N^6-benzyl-adenine; NAA, naphthalene acetic acid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase, high performance liquid chromatography; UV, ultra-violet; IR, infra-red; DEAE, diethylaminoethyl; QAE, diethyl-(2-hydroxy-propyl)-aminoethyl.

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tion. Delphinidin-3-rutinoside (tulip), cyanin (rose), pelargonin (salvia), and betanin (red beet) were gifts. Cyanidin chloride, cyanin chloride, pelargonidin chloride, and pelargonidin chloride 3-glycoside were from Funakoshi Yakuhin Co., Ltd. (Tokyo, Japan). α-Carotene and casein hydrolysate were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). β-Carotene, cellulose, and silica thin-layer plates were from E. Merck (Darmstadt, F.R.G.). Agar, starch (potato), pectin (citrus), cyclodextrin, inulin, maltose, lactose, fructose, glucose, galacturonic acid, gelatin, *p*-fluorophenylalanine, glyphosate, and polypeptones were from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Cellulose powder was from Tokyo Roshi Co., Ltd. (Tokyo, Japan). Chitin, Chitosan, and methyl cellulose were from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Bactosoyton and yeast extract were from Difco Laboratories (Detroit, Mich., U.S.A.). DEAE-Sephadex A-25 and QAE-Sephadex A-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals and reagents used were of analytical grade or the best grade obtainable commercially.

Initiation of callus cultures

Seeds of *C. tinctorius* were grown in pots in a green house at about 28 °C for about 3 months. Capitula from the adult plant were gathered at random just before blooming. These were immersed in 2% antiformin/0.5% Tween-20 solution for 10 min, after which they were rinsed three times in sterile water. Extraneous materials were removed, and the meristem tissues excised axenically from the capitula with a pincette and small dissecting knife under a microscope. The separated meristems were placed on LS agar medium in which the nutrient composition had been slightly modified. This growth medium was supplemented with the following additional nutrients (mg/1000 ml): Thiamine-HCl (0.1), pyridoxine-HCl (0.5), nicotinic acid (0.5), glycine (0.2), sucrose (30000), NAA (0.19), and BA (0.23) (KBC-G medium). Cultures were incubated at 25 ± 1 °C in the dark. After about 3 weeks, the excised segments had formed relatively uniform callus.

Isolation and culture of cell cultures

Readily friable callus pieces with a combined fresh weight of 2–3 g were placed in 300 ml flasks containing 75 ml of fresh liquid medium of the same composition as the callus medium, but without agar. When a thick suspension of callus and small clumps had formed, the clumps were removed to fresh medium, giving a starting packed volume of 30–40%. After a week, 15 to 20 ml portions of this cell suspension were transferred to flasks containing fresh medium. This was done every 3 to 4 days to enrich the rapidly growing cells. The *Carthamus* cells were then subcultured routinely at about a 10-fold dilution at 6- to 8-day intervals. The culture flasks were agitated on a rotary shaker at 100 rpm and 25 ± 1 °C in darkness.

Induction of pigment synthesis in cell suspension cultures

The small cell aggregates that formed (approx. 3.5 g wet wt.) were transferred to 75 ml of fresh liquid medium in 300 ml flasks containing (if not otherwise indicated) the additional components, D-phenylalanine (165.2 mg/1000 ml) and cellulose powder (40 g/1000 ml) (KBC-P1 medium), and again were agitated on a rotary shaker at 100 rpm and 25 ± 1 °C in the dark.

Extraction and purification of a red pigment

After 3 days, the cultured suspension was sieved through a stainless steel, 149 μm pore screen, after which the transit liquor was filtered through a Büchner funnel. The cellulose residue on the funnel was washed thoroughly with distilled water, then dried in a vacuum freezer and stored in a flask at −20 °C. The air in the flask was evacuated and replaced by N_2 gas. This cellulose stock was suspended in 5 times per dry wt. of acetone/methanol (1:1, by vol.), and the suspension centrifuged at about 3500 × *g* for 10 min. The supernatant was retained and the precipitate resuspended in the same volume of acetone/methanol (1:1, by vol.), then centrifuged. The extracts obtained were pooled and the precipitate was treated once again with fresh solvent to extract the pigment. The extracts were combined and evaporated *in vacuo* below 30 °C, which gave a dark reddish brown amorphous mass. This mass was separated in an RP-HPLC system.

HPLC analyses were done with a Shimadzu liquid chromatograph, type LC-6A equipped with a SPD-6AV UV-visible variable wave-length detector and a C-R 3A chromatopac data station. A reverse phase column composed of 5 μm particles was used

(250 × 10 I.D.). The working solution contained approximately 0.1 mg of sample per 2 ml of 90% methanol. The elution solvents were 65 and 90% aqueous methanol. Runs were made for 15 min at 62–65 atm. The flow rate was 1.8 ml/min. A 150- to 200-µl sample was administered with a micro-injector, and the column flushed with a linear gradient of the elution solvents. Peaks were detected at 254 and 520 nm. The combined column eluates were concentrated below 30 °C with a rotary evaporator. This condensed matter was analyzed by preparative RP-HPLC or was recrystallized in aqueous acetone-methanol.

Analysis of the red pigment

The powdered micro-crystalline red pigment was compared with authentic samples of carthamin, anthocyanins, betanin, and carotenoids. Co-chromatography was done on cellulose or silica thin-layer plates with the following six solvent systems: (A) 1-Amylalcohol/acetone/water (5:6:5, by vol.), (B) ethyl acetate/pyridine/water (14:5:3, by vol.), (C) 1-butanol/pyridine/water (6:4:3, by vol.), (D) 1-butanol/acetic acid/water (4:1:2, by vol.), (E) acetic acid/water (15:85, by vol.), and (F) phenol saturated with water. The colours and R_f-values of the purified sample and the standard specimens were compared on air-dried chromatograms under UV or visible light, some plates being exposed to chromogenic reagents such as ammonia vapour or alcoholic $FeCl_3$ solution. Some samples were mixed in vials with conc. HCl-powdered Mg, HCl or H_2SO_4, and the fine colour change compared with those of authentic compounds used. UV spectra were measured at 200–600 nm in methanol with a Shimadzu, type UV 150-02 spectrophotometer. IR spectra were recorded with a Nippon Denshi, model JIR-100 spectrophotometer on micro KBr disks at a scan speed of 8 mm/s.

Measurement of pigment content and the weight of the cultured cells

At the end of the desired incubation period, the cellulose powder was separated from its culture medium by sieving the suspension through a 149 µm micro-pore screen, after which the residue was washed many times on a Büchner funnel with deionized-distilled water. The damp cellulose obtained was treated with pyridine. The pigment content of the pyridine extract was determined by spectrophotometry in terms of the carthamin concentration in the pyridine solution. The specific value of the absorbance was expressed as the relative rate at 520 nm in the test solution to that in the control of accumulation (1.0).

Fresh weights of the cell cultures were obtained from the damp cell aggregates that remained on the Büchner funnel. Dry weights were determined as follows: weighed wet cells (approx. 20 g) were dried in a Mitamura circulation oven, type 1037 at 80 °C until the weight became stationary (2 to 2.5 days). Before the weight determination, the dried cells were placed in a desiccator over silica gel for at least 1 h. Measurements were made at 25 ± 1 °C in an air-conditioned room.

Results

Formation of a red pigment by C. tinctorius cell suspension cultures

In a preliminary experiment, rapidly propagating cell aggregates were transferred to various culture media such as MS [8], LS [9], White [10], and Gamborg *et al.* [11]. After a given period of culture, extracts of the cellulose powders separated from the culture media or from callus tissues on agar media were assayed for pigment. Red pigment was present only in the extracts from tissues cultured on White's medium, a medium similar to KBC-P medium, especially in its inorganic nutrient composition.

The cultured *Carthamus* cells excreted a red pigment into the liquid culture medium, which was trapped by the addition of cellulose powder. The average content of this red pigment, in terms of carthamin, was roughly 51 pg/mg per wet cell/h. The time course of the red pigment accumulation and the changes in the weight of the cultured cells are shown in Fig. 1. The lag in the pigment synthesis that lasted more than half a day after transfer of the *Carthamus* cultures to the pigment-generating medium (KBC-P1 medium) was followed by a linear increase in the red pigment content during the last 2.5 days. Moreover, the cell cultures continued to increase the amount of the pigment produced until the 3rd day of culture, after which production declined sharply and became stationary.

The fresh weight increment shows that there are two phases in the growth of *C. tinctorius* cell cultures: after a short lag phase of an hour, the cultures proliferated two peaks occurring during this growth

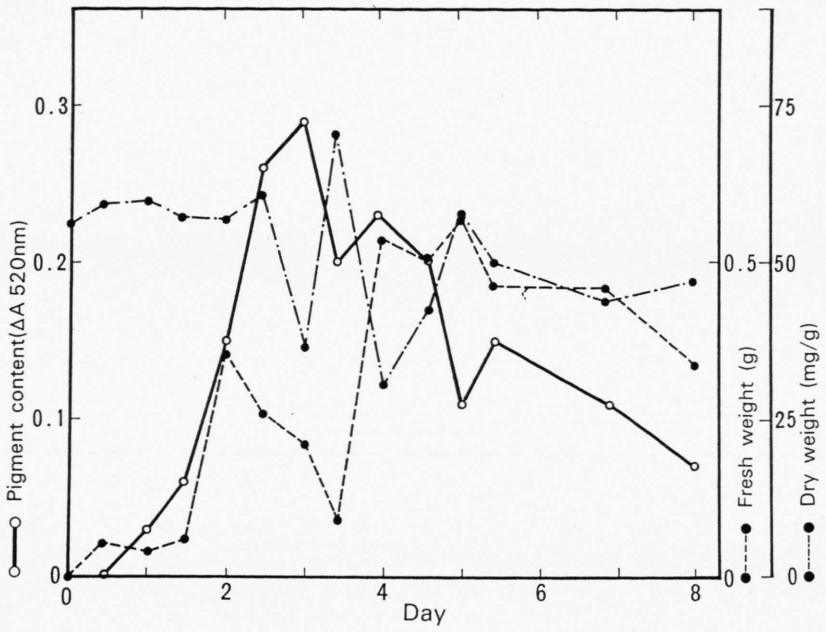


Fig. 1. Time course of pigment accumulation and weight change in the cultured cells of *C. tinctorius*.

period. The first peak appeared 2 days after inoculation as a sharp increase and was followed by a rapid decrease in cell weight (3.4 days later). The second increase took place 4 to 5 days later, after which growth stopped and there was a linear decrease. In contrast, the dry weight remained stationary up to 2.5 days after incubation, then decreased linearly with incubation time. This may be because no *de novo* synthesis of protein occurred in cultured cells transferred to KBC-P1 medium, whereas, the fresh weight increased. The decrease in the dry weight is correlated with the decrease in the pigment content.

General characteristics of the red pigment

Both currently isolated pigment and an authentic carthamin are red on thin-layer plates, but they have different R_f-values on the same plates developed with several solvent systems. The values for the pigment and carthamin in solvents (A), (B), and (C) were 0.56:0.61, 0.55:0.42, and 0.72:0.78. R_f- and R_t-values of the two samples for reverse phase TLC and HPLC were: TLC = 0.99:0.50 and HPLC = 5.83:10.19. UV spectra of the pigment from the cell cultures were: λ_{max}^{MeOH} 208, 485, and 517 nm and of standard carthamin 205, 270, 369, and 517 nm. Elemental analysis of the isolated pigment gave C = 47.9, H = 4.8 and of carthamin C = 64.4, H = 5.7. The pigment is soluble in water, methanol, ethanol, ethyl acetate, and acetone. Thermally it is more unstable than carthamin in aqueous solutions. It is degraded irreversibly at room temperature within a few days into as yet undetermined orange-yellow or light-yellow substances. It reacts negatively with ammonia, H_2SO_4, HCl and HCl-powdered Mg, and is not soluble in petroleum ether. Comparison of data from the IR spectra of the novel red pigment and of authentic carthamin show that they clearly differ (Fig. 2A and 2B).

The colour and chromatographic behaviour of the pigment on cellulose or silica gel plates different markedly from those of anthocyanins, betanin, and carotenoids (data not given). UV spectral data also indicate that the isolated pigment is not identical with any of the other plant pigments examined (Fig. 3A–3D). Structural analysis of this new pigment is now being done in our laboratories.

Increasing the pigment content by modifying the culture media

Rapidly growing cell aggregates were transferred to media with or without one or more groups of constituents (micro-elements, macro-elements, sub-

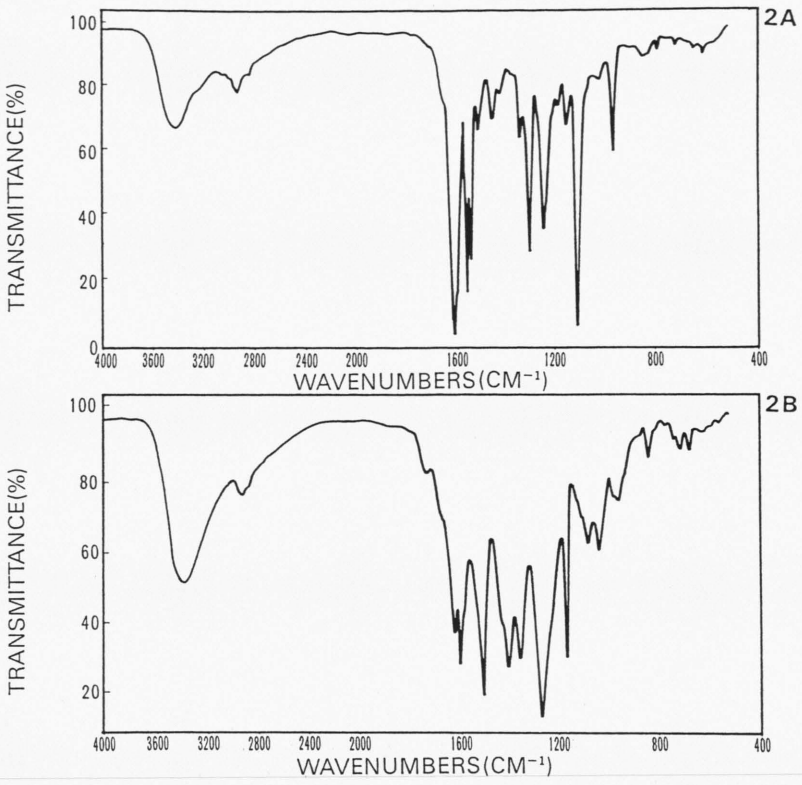


Fig. 2. IR spectra of (A) newly isolated pigment and (B) carthamin.

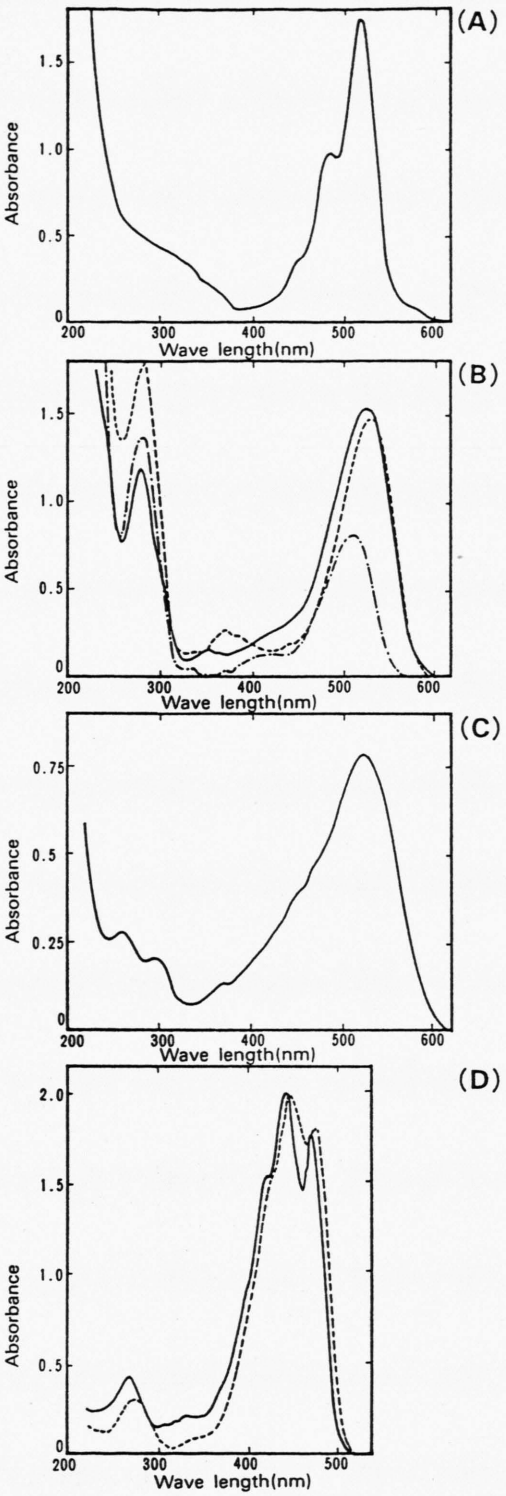
strates) to see which affected the pigment content. Reduced anion and cation contents in KBC-P and KBC-P1 (KBC-P + 1 mM D-phenylalanine) media strikingly affected the pigment synthesis in the cultured cells (Fig. 4A and 4B). Omission of Mg^{2+} markedly increased the amount of the pigment, about 1.2-fold in KBC-P and about 18-fold in KBC-P1 media, respectively. Removal of Ca^{2+} also influenced the pigment formation, but not as much as Mg^{2+}. About a 5-fold greater pigment content was found in the cell cultures grown on KBC-P1 medium.

Cells grown on the media without NO_3^- produced no pigment. Negligible amounts of red pigment were synthesized by cells cultured on the media deficient in NH_4^+ and PO_4^-. When these ions were omitted together, there were further pronounced decrease in the pigment. The concurrent removal of NH_4^+–Mg^{2+}, NH_4^+–Ca^{2+}, Mg^{2+}–Ca^{2+}, and NH_4^+–Mg^{2+}–Ca^{2+} from the media produced respectively (fold for the control, KBC-P:KBC-P1) 1.6:22.3, 0.6:4.5, 0.8:23.7, and 1.2:23.1. Clearly, these ions have a key function in the synthesis of the novel red pigment produced in *Carthamus* cell suspension cultures.

Effect of possible precursors on pigment formation

Effect of possible precursors for flavonoid synthesis (kinate, shikimate, β-phenylpyruvate, phenylalanine, tyrosine, tryptophan, cinnamate, and *p*-coumarate) were determined separately in the concentration range from 0.001 to 10 mM. The effect of D-, L-, and DL-phenylalanine on the rate of pigment synthesis are shown in Fig. 5A. D-Phenylalanine is a positive stimulator, but the L-form is negative. L- and D-tyrosine are both inert (Fig. 5B). L-, D-, and DL-tryptophan are all effective promoters. The L- and D-forms are the strongest at 1 mM, whereas, the DL-form affects the pigment synthesis at a concentration higher than 10 mM (Fig. 5C).

The effects of four organic acids were compared at various concentrations (Fig. 5D). Of these only kinate was promising. It stimulated pigment produc-



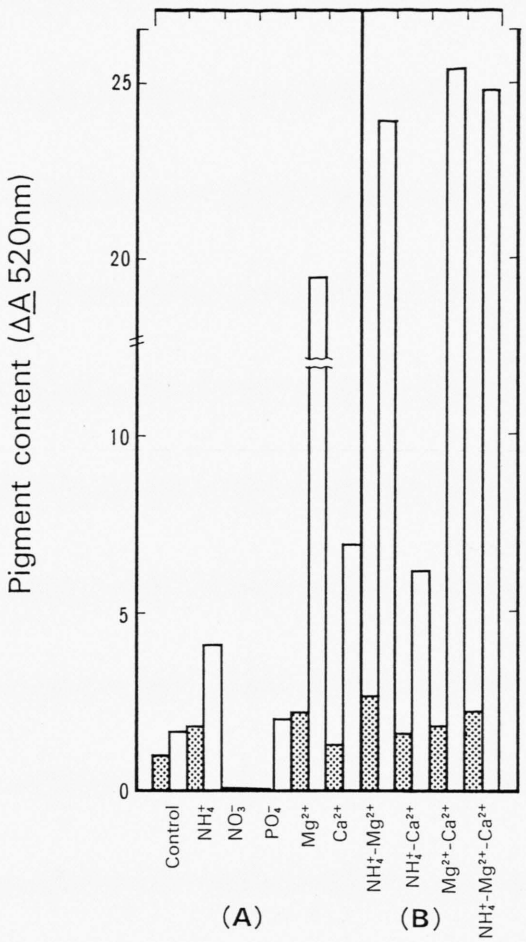


Fig. 4. Effect of micro-elements on the formation of a novel red pigment by cultured cells from the meristem tissues of *C. tinctorius*. (A) A single element was omitted, (B) multiple elements were omitted. ⊡: Cell cultures grown on KBC-P medium, □: cell cultures grown on KBC-P1 medium.

Fig. 3. UV spectra of (A) newly isolated pigment, (B) anthocyanins, (C) betanin, and (D) carotenoids. (B) ———: delphinidin-3-rutinoside; – – –: cyanin; —·—·—: pelargonin, (D) ———: α-carotene; – – –: β-carotene. The UV spectra of the two carotenes were measured in hexane.

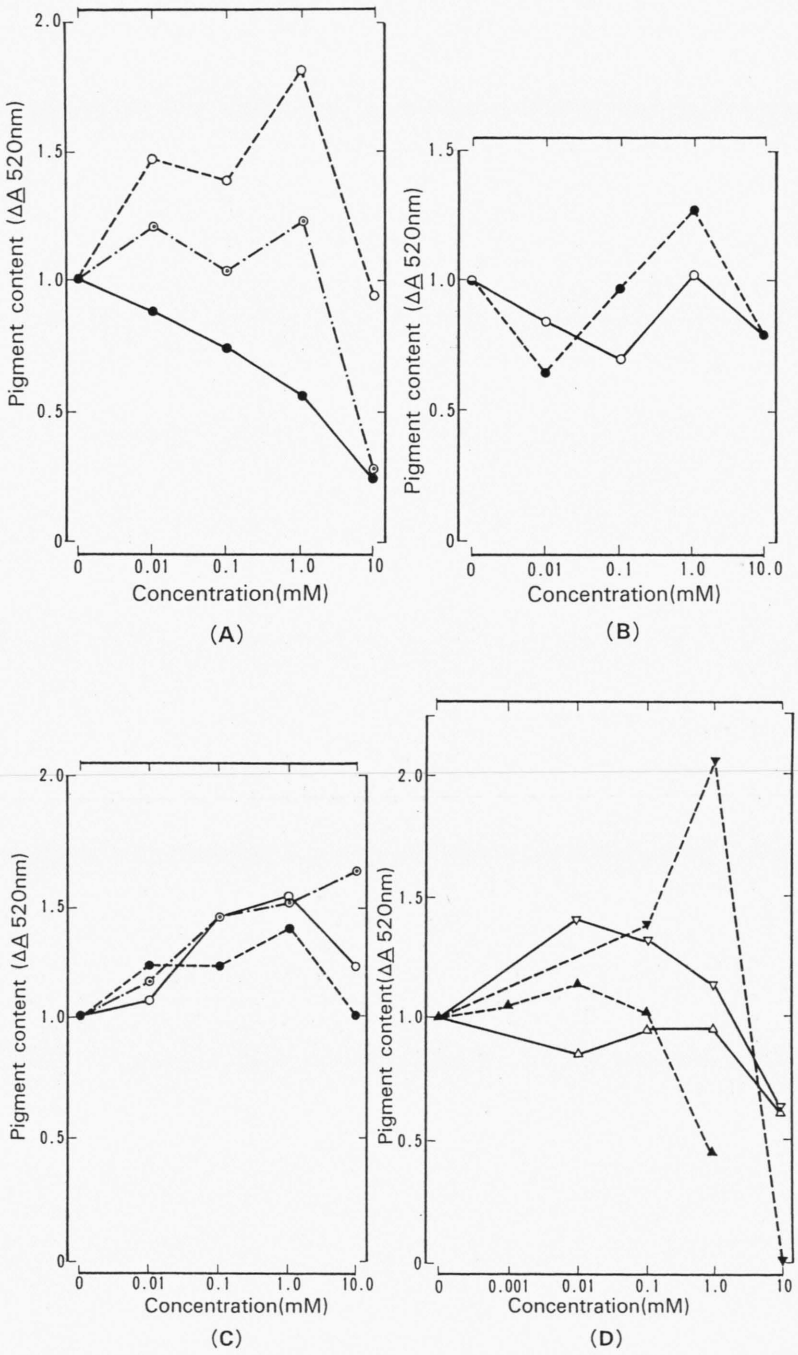


Fig. 5. Effect of putative precursors on pigment formation by cell suspension cultures of *C. tinctorius*. (A) Effect of phenylalanine; ●−−−●: **L**-form, ○−−−○: D-form, ⊙−−−⊙: DL-form. (B) Effect of tyrosine; ●−−−●: L-form, ○−−−○: D-form. (C) Effect of tryptophan; ●−−−●: L-form, ○−−−○: D-form, ⊙−−−⊙: DL-form. (D) Effect of organic acids; △−−−△: β-phenylpyruvic acid, ▲−−−▲: *trans*-cinnamic acid, ▽−−−▽: *p*-coumaric acid, ▼−−−▼: kinic acid.

tion markedly at the concentration of 1 mM. This acid may be used as a substrate by the cultured cells during the synthesis of the novel red pigment, but it is not clear why D-phenylalanine has such a marked effect on the pigment formation.

Effect of macro-molecular compounds on pigment production

A cell strain dormant for the production of a red pigment was transferred to flasks containing KBC-P medium supplemented with different amounts of

various macro-molecular substances. The compounds had different effects on the pigment formation (Table I). The mechanism that causes these influences has yet to be determined.

Table I. Effect of various substances on pigment formation by callus tissues and cell suspension cultures of *C. tinctorius*.

Compound	Concentration [%]	Pigment produced	
		Callus	Cultured cell
Control	–	–	–
Glucose	1	–	–
Fructose	1	–	–
Lactose	1	–	–
Maltose	1	–	–
Galacturonic acid	1	–	–
Inulin	1	–	–
Cyclodextrin	1	–	–
Starch (potato)	1	–	–
Cellulose	1	±	++
Methyl cellulose	1	–	–
Cotton	–*	–	+
Tissue paper	–*	±	+
Chitin	1	±	++
Chitosan	1	++	+++
Pectin (apple)	0.5	–	+
Pectin (citrus)	0.5	–	+
Gelatin	0.5	–	–
Milled rice	1	±	–
Agar	0.5	–	–
DEAE-Sephadex A-25	1	–	–
	2	–	–
QAE-Sephadex A-25	1	–	–
	2	–	–
p-Fluorophenylalanine	0.01	–	–
Sodium arginate	0.5	–	–
Glyphosate	0.5 (ml/1000 ml)	–	–
Polypepton	0.1	–	–
Bactosoyton	0.1	–	–
Yeast extract	0.1	±	–
Casein hydrolysate	0.1	–	–
Amberlite CG-400	1	–	–
	2	–	–
Dowex 1-X2	1	+	+
	2	+	+
Dowex 2-X8	1	+	+
	2	–	–
Aluminium oxide	1	–	+
Quartz wool	–*	–	–
Silica gel	1	–	–
Glass beads	1	–	–

* Amount was not clear. Pigment was assayed in aqueous extracts from 1- to 5-day-old material after inoculation on KBC-P medium supplemented with test substances at the concentration indicated in the table. –: pigment was not detected, ±: the presence of the pigment was not clear, +: 5–40 μg/ml pigment was detected, ++: 41–80 μg/ml pigment was detected, +++: 81–120 μg/ml pigment was detected.

Discussion

A cell strain obtained from excised meristem callus cultures of young developing capitula of *C. tinctorius* produced a substantial amount of a novel red pigment in the dark in liquid culture medium supplemented with specified quantities of phytoregulators, macro- or microelements, and putative substrates. The pigment was obtained as a microcrystalline powder by repeated chromatography and subsequent recrystallization. It is royal red in aqueous methanol and in acetone, as is carthamin, but it migrates differently on TLC and HPLC as shown by its specific R_f- and R_t-values. Analytic data on the element in its composition and its UV and IR spectra are all clearly different from those of authentic carthamin. The results from preliminary tests also indicated that it differs from anthocyanins, betanin, and carotenoids.

We have tentatively named this novel reddish pigment "Kurenamin", and are now characterizing its structure. Although red carthamin is normally accumulated in fully expanded and matured tube flowers of *C. tinctorius,* cultured cells from the younger meristems showed strikingly different pigment productivities, even though they were considered as having the same genotype as the parent plant.

Variation in the yields of plant pigments have been reported in callus and in cell suspension cultures of various plant materials [12–14]. Alfermann and Reinhard [15] isolated anthocyanin-producing and anthocyanin-free strains from callus cultures of *Daucus carota.* Sugano *et al.* [16] succeeded in isolating two different strains from a red carrot, one that primarily produced β-carotene and the other lycopene. Cell cultures of *Centaurea* stem produced a malonated cyanidin glucoside which occurs in the leaf, but not in the flowers, of the parent plant [14].

The pigment productivity of *Carthamus* cell suspension cultures differs from those of *Daucus* and *Centaurea.* Our cell line does not have the ability to synthesize and accumulate normal red carthamin. Instead, it produces a novel red pigment which we have tentatively named kurenamin, and which has not yet to be isolated and characterized from the parent *Carthamus* tissues. This suggests that our cultured cells have lost certain inherent properties during prolonged culture on artificial media and, therefore, produce a different type of reddish pigment. The

specific ability to synthesize this novel pigment must be triggered through changes in various conditions in the medium and of culture. We have still to clarify the mechanism for the synthesis of kurenamin.

Strains, even those from a single genotype, differ phenotypically. This heterogeneity has been attributed to the fact that different parts of plant tissues have different potentials for the accumulation of secondary metabolites and/or different activities for some of the enzymes responsible for metabolite formation [7]. But, it has also been postulated that if the synthesis and the subsequent accumulation of a particular secondary product is, in any way, dependent upon a specialized cellular structure, a plant cell culture can only be exploited for metabolite production if the same structural modification can be induced [17].

Many secondary metabolites, including phenolic pigments, are formed only in highly specialized organs such as roots, leaves, and flowers. Cosmocyanin of *Cosmos* is found mainly in the flower petals of pink- and crimson-coloured cultivars [18]; betalains are found mainly in the flowers, fruits, and leaves of the Centrospermae [19]; and shikonin is synthesized principally in the roots of *Lithospermum erythrorhizon* Sieb. *et* Zucc. [20]. Therefore, during plant growth and maturation, cells must undergo not only morphological specialization, but differentiation of their abilities to produce specific metabolites. In cell suspension cultures this type of differentiation is absent; therefore, product synthesis is believed to be suspended or extremely diminished.

Several questions have yet to be answered. Is carthamin synthesis induced only in highly specialized tissues or organs? Why is no kurenamin detectable in intact meristem tissues of *C. tinctorius?* What regulatory mechanism controls the synthesis of this novel pigment? How is kurenamin induced in the cultured cell aggregates? Further detailed studies are needed to furnish the answers to these important questions. We are now performing experiments for the detection of kurenamin and its precursors in extracts from the intact florets and from the cell suspension cultures of *C. tinctorius*.

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