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The enzymatic mobilization of bound precarthamine from the flower florets of *Carthamus tinctorius* L.

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Abstract

Floret pieces prepared from the methanol-treated and air-dried flowers of safflower (*Carthamus tinctorius* L.) were subjected to an enzymatic hydrolysis in a buffered solution and the rate of precarthamine solubilizalion was assessed spectrophotometrically. The amount of precarthamine was risen by treating the floral pieces with a β-glucosidase (EC 3.2.1.21), supporting a previous assumption that precarthamine is in the floral tissues associated with cellular components. The enzyme activity was reduced by various inhibitors. On the basis of the experimental data, it is proposed that the precarthamine may be bound through *O*-β-glucosyl linkage(s).

Introduction

Previously, we have demonstrated that precarthamine is less solubilized in aqueous organic layers than other similar pigments such as safflor yellow A and safflor yellow B. The solubility of precarthamine rose to a great degree after trituration of fresh florets from *Carthamus tinctorius* capitula, which led us to suppose that the pigment may be chemically bound to cellular components (Saito and Katsukura 1993). Lately, we have observed that the pigment liberation is accelerated considerably by the addition of aqueous acetone (Saito 1993). However, the mechanisms have been unrevealed.

To substantiate the tentative postulation, further works on the chemical interaction between precarthamine and cellular components were continued in the present study.

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Abbreviations: BAW - *n*-butanol/acetic acid/water (4 : 1 : 2, v/v); HOAc - acetic acid/water (15 : 85, v/v); IEW - *i*-propanol/ethylacetate/water (6 : 3 : 1, v/v); MW - methanol/water (6 : 4, v/v); AW - acetone/water (6 : 4, v/v); TLC - thin-layer chromatography; PC - paper chromatography; UV - ultraviolet radiation; VIS - visible radiation.

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Materials and methods

Materials: Precarthamine and carthamine used as authentic markers were from the same source as described previously (Takahashi *et al.* 1984). β-Glucosidase (EC 3.2.1.21) from almond (specific activity 13.7 units mg^{-1}), *D*-(+)-glucosamine-HCl, *p*-(hydroxymercuri)benzonate, iron(III) sulphate and silver potassium cyanide were obtained from *Wako Pure Chemical* (Osaka, Japan). Copper(II) sulphate was purchased from *Nakarai Tesque* (Kyoto, Japan). Silica and cellulose TLC plates were purchased from *Merck* (Darmstadt, Germany). PC sheet No. 50 was supplied by *Advantic Toyo* (Tokyo, Japan). *Avicel* cellulose was obtained from *Asahi Kasei Kogyo* (Tokyo, Japan). Other chemicals and reagents used were all analytical grade of purity obtainable commercially.

The freshly picked florests of field grown *C. tinctorius* were dipped in glass distilled methanol for about 1 week at 4 - 5 °C in the dark to inactivate the endogenous enzymes. On filtrating methanol off, the florets were dried in an air-circulation oven and kept in a freezer at -20 °C just before the experimental use.

Treatment of florets with glucosidase: Before conducting the enzyme experiments, aliquots of the dried florets were crushed into small pieces with a pestle and mortar. The powdered florets (500 mg each) were suspended in 10 cm^3 of 50 mM citrate buffer, pH 4.0 containing 0.1 mg β-glucosidase sample. The suspension in a Thumberg tube was evacuated 5 times with an aspirator and air in the tube head space replaced with dinitrogen, then it was kept at 30 °C for given intervals by gentle agitating at 100 strokes min^{-1} in a water-bath incubator.

Location of precarthamine in the enzymatic hydrolysate: At the end of the incubation, 0.01 cm^3 glacial acetic acid was mixed into the reaction medium to stop the enzyme activity. An aliquot of the medium was transferred to a plastic tube and centrifuged at 4000 *g* for 5 min. The clear supernatant was spotted on PC sheets or on silica and cellulose TLC plates, then they were developed in BAW, HOAc or in IEW.

After drying the chromatograms were sprayed with 50 μM $KMnO_4$ (Saito 1991) and appeared red spot was marked with a pencil. When required to compare the R_f-values and the colour with those of an authentic standard, the chromatograms were often examined closely under an UV lamp (365 nm) or in the day light.

Isolation and identification of released precarthamine by glucosidase hydrolysis: Precarthamine was extracted with ethylacetate. The condensed extract was chromatographed on glass columns of *Avicel* cellulose (4.7 × 51.6 cm) and (3.3 × 48.5 cm) or a *Toyo Pearl column* (1.7 × 30.5 cm) with the developing solvents, BAW, IEW and MW, respectively. An aliquot (10 cm^{-3}) of the partially purified precarthamine was injected onto a *Wakosil* $5C_{18}$ high-performance liquid chromatographic column (4 × 250 mm, 5 μm) fitted with a *Jasco* eluent supply and a *Jasco* UV/VIS detector. The solvent AW was delivered at 0.3 cm^3 min^{-1}. Absorbance at 410 nm was monitored and the area of the absorption peak corresponding to precarthamine was compared to a standard curve generated with authentic specimen.

Determination of enzyme-mobilized precarthamine content: The content of precarthamine released by enzymatic hydrolysis was determined indirectly. This was done following the method of Saito *et al.* (1992) with slight modification. The glucosidase treated floret (250 mg dry wt) suspension was centrifuged at 4000 *g* for 5 min and the supernatant sucked out. The pellet was washed 2 - 3 times with water through centrifugation (4000 *g*, 5 min). To the pooled supernatant (250 cm^3), 5 cm^3 of 0.5 mM potassium permanganate was mixed by stirring for 2 - 3 min, then *Avicel* cellulose (1 g) was suspended and kept for several minutes with gentle stirring on a magnetic stirrer. The suspension was collected by filtration on a Büchner funnel and the residue washed 3 times with water (300 cm^3 in total). The red *Avicel* thus prepared was suspended again in 25 - 30 cm^3 of 60 % (v/v) acetone, stirred for 5 min and centrifuged at 4000 *g* for 5 min. The acetone layer was pooled and the extraction repeated 3 - 4 times, at each repetition, old 60 % acetone was replaced by fresh one. The retained acetone extracts were taken up to a net volume (100 cm^3) and applied to the process of the spectrophotometric determination of the enzyme-mobilized precarthamine content. A *Hitachi*, model *U-1100* spectrophotometer was used with 60 % aqueous acetone as reference. The data from the spectrophotometric reading at 521 nm were referred with a calibration curve. The amount of precarthamine, whose chemical structure remains obscure, was calculated in terms of carthamine (M_r = 910) equivalent.

Administration of chemicals: *D*-(+)-Glucosamine, *p*-(hydroxymercuri)benzoate, iron (III) sulphate, copper(II) sulphate and silver potassium cyanide were dissolved separately in distilled water to obtain 1 mM concentration. 1 cm^3 of each solution was added to the reaction medium containing 500 mg powdered florets, 0.1 mg β-glucosidase and 9 cm^3 of 50 mM citrate buffer, pH 4.0, and incubated at 30 °C for 3 h. At the end of enzymatic reaction, the effect of the external additives on the enzyme activity was estimated by the release rate of precarthamine following the indirect method as described above.

Results and discussion

Precarthamine (Fig. 1) is released by the process of enzymatic hydrolysis. Precarthamine content rose with time of the action of β-glucosidase (Fig. 2A). Under the condition of the present study, 3 h incubation was the most effective and, at this period, precarthamine was released with a velocity of 0.119 nmol(precarthamine) cm^{-3} min^{-1}. In shorter and longer intervals of the incubation, the recovery rate of precarthamine was reduced considerably. Thus a 3 h incubation time was used in this study, where 50 mM citrate buffer was exclusively applied at pH 4.0 (Fig. 2B). The enzymatic process was sensitive on various inhibitors: the test chemicals fed in 1 μmol concentration reduced the rate of precarthamine dissociation by 13.5 - 25.9 % of the control (Table 1). These data endorse strongly the fact that precarthamine is bound to cellular components through *O*-β-glucosyl linkage(s).

Flavonoids and related compounds are generally supposed to exist in the vacuoles and/or in the cell sap of plant tissues at free state. Indeed, safflor yellow A and B,

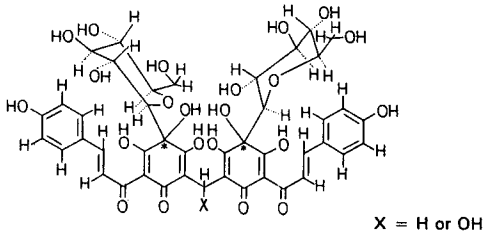

Fig. 1. A postulated structure of precarthamine.

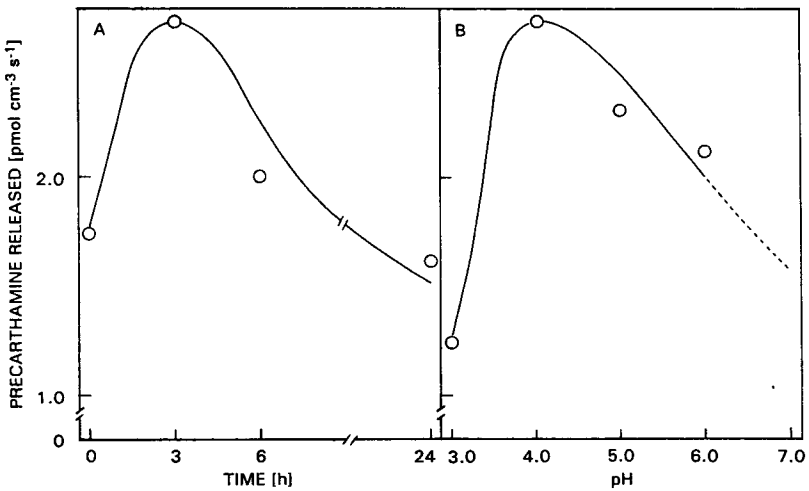

Fig. 2A. Time-course of precarthamine release by β-glucosidase hydrolysis. The incubation was carried out with 500 mg floret powders and 0.1 mg β-glucosidase in 10 cm^3 of 50 mM citrate buffer, pH 4.0.

Fig. 2B. The effect of pH on precarthamine release by β-glucosidase hydrolysis. Enzyme activity was measured in 50 mM citrate buffer at various pHs as indicated. For details of the incubation systems and assay conditions, see Materials and methods.

synonymous chalcoquinoid glycosides with precarthamine are readily extractable with water or aqueous organic solvents. Perhaps, they occur in arresting loosely or at free state in the floral parts of *C. tinctorius*, though their locus and compartmentation are still uncertain. Thus the present data suggest that two types of chalcoquinoids are produced in *Carthamus* flowers, the one is bound type and the others are free types.

In case of plant tanins, two groups are found: hydrolyzable tanins and condensed ones, the former being consisted of lower molecular mass than the latter. Pentagalloyl glucose, the simplest hydrolyzable tanins is a conjugate of gallic acid. Corilagin, another hydrolyzable tanin, is linked to phenolic groups to render the molecules more water-soluble and ensure their sequestration in the vacuole. These

appreciable characteristics of tannic compounds may be in conformity with precarthamine or with safflor yellow A and safflor yellow B.

Table 1. Effect of inhibitors on the precarthamine release by β-glucosidase catalysis.The reaction was carried out under the standard assay condition as shown in Materials and methods.

Inhibitor	Rate of precarthamine release [pmol cm^{-3} s^{-1}]	[% of control]
None	1.983 ± 2.133	100
D-(+)-Glucosamine	1.717 ± 0.132	86.6
Silver potassium cyanide	1.550 ± 0.087	78.2
p-(Hydroxymercuri)-benzoate	1.500 ± 0.013	75.6
Copper(II) sulphate	1.500 ± 0.080	75.6
Iron(III) sulphate	1.467 ± 0.067	74.0

The bound precarthamine, inclusive of free safflor yellow A and B, plays most likely a significant role in the physiological control of the flowering. The continuation of systematic work can find the role of these compounds in the regulatory mechanism.

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