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Sono-oscillatory mobilisation of bound precarthamine from flower florets of dyer's saffron

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Floral pastes of dyer's saffron (*Carthamus tinctorius* L.) were subjected to machinery vibration by an ultrasonicator in the presence of α- or β-glucosidase and the rate of precarthamine release was examined spectrophotometrically. On sono-oscillating the pastes at 450 kHz for 10 min at 25 ± 1°C, the release of bound precarthamine was promoted by 19·9% in the presence of α-glucosidase. The supersound effect on the precarthamine release by β-glucosidase was also pronounced, showing a net increment rate of 20·3% on average. The usefulness of supersonic oscillation for obtaining oxygen-labile and pH-sensitive precarthamine was assessed on the basis of the experimental results.

INTRODUCTION

There is a general recognition that flavonoids are accumulated in vacuoles in the free state in vascular plant tissues. However, our recent findings are clearly at variance with the widespread belief that (a) precarthamine, a quinoidal chalcone pigment, is less extractable with organic solvents; (b) it is obtained with strong acid mixtures such as methanol/HCl, dilute trifluoroacetic acid, or aqueous trichloroacetic acid; (c) trituration of dyer's saffron flowers elevates precarthamine yield (Saito & Katsukura, 1993); and (d) a hydrolytic enzyme treatment of the floral pastes is very useful to improve precarthamine recovery rate (Saito & Yamamoto, 1994). These results lead us suppose that precarthamine may be bound tightly with cellular components in the floral tissues.

The dye is very sensitive to atmospheric oxygen and changes readily to red carthamine (Fig. 1). It is unstable, like most flavonoids in solutions in strong acids or bases. The instability of precarthamine makes it, technically difficult to obtain purified samples in abundance. At present, we use β-glucosidase from yeast to extract precarthamine from dyer's saffron flowers under mild conditions (Saito, 1993), because the conventional method with methanol/HCl mixtures is very troublesome, in particular, in the acid mixtures listed above (b) there is difficulty removing traces of HCl: we are always compelled to spend long hours (which should be as short as possible) before purifying the oxygen-sensitive and solvent-labile quinoidal chalcone glycoside. Although the new enzymatic procedure for extracting precarthamine frees us from the troublesome work and raises pigment yields, better techniques would be of use to prepare ample precarthamine dye more quickly.

In our preceding works we have introduced new procedures to hasten enzymatic and non-enzymatic reactions, where peroxidase-catalysed carthamine bleaching and oxidizing agent-mediated carthamine production are enhanced by supersonic oscillation treatments (Saito *et al.*, 1991; Saito & Miyakawa, 1994).

The relevant method will be assessed to see if supersonication could raise precarthamine yields, and to determine the chemical structure of precarthamine and establish the practical utility of the oxygen-sensitive and solvent-labile quinoidal chalcone dye. This prework is of great importance in assessing the potential for use of the pigment as a food colorant.

MATERIALS AND METHODS

Materials

α-Glucosidase (EC 3.2.1.20, specific act. 50·6 units/mg) and β-glucosidase (EC 3.2.1.21, specific act. 36·0 units/mg) from yeast were obtained from Oriental Kobo (Tokyo, Japan). Tubular flowers were collected from the freshly opened flowering heads of dyer's saffron on 3–5 August 1993. They were plunged into boiling methanol and kept for 5 min. The denatured flowers were dried in an air-circulation oven at 30°C

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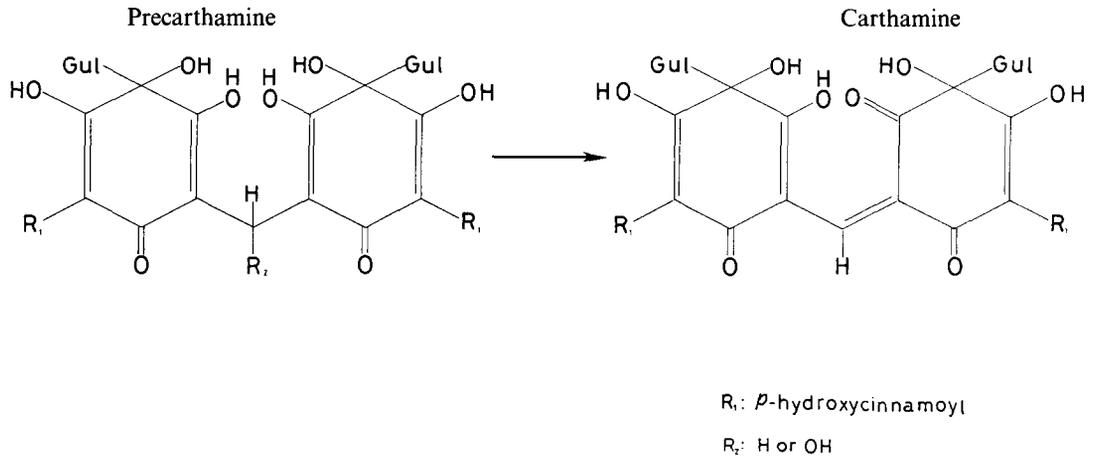

Fig. 1. A supposed route from precarthamine to carthamine.

for 7–8 h and ground into small pieces in a porcelain mortar with a pestle.

Extraction of free precarthamine

An aliquot of powdered flowers from the above section was suspended in 60% (v/v) methanol and stirred on a magnetic stirrer for 60 min at room temperature. The suspension was transferred to Teflon tubes and centrifuged at 4000 × *g* for 5 min. The supernatant was replaced by fresh 60% methanol and centrifuged again (4000 × *g*, 5 min). The methanol washing was continued a further four times. The free precarthamine (removed) flower pellets were dried in the air and stored at –20°C in the dark just before use.

Administration of glucosidases

Flower powders, from which free precarthamine had been removed (0·5 g each), were suspended in 10 ml 50 mM citrate/phosphate buffer, pH 6·8 (α-glucosidase) or in 10 ml 50 mM citrate buffer, pH 4·5 (β-glucosidase). Each suspension in a 30 ml beaker was placed in an oscillatory chamber of a sono-sound oscillator, Bransonic, model 1200 (Yamato, Tokyo) and sonicated at 450 kHz for 10 min at 25 ± 1°C. At the end of the incubation, the enzyme reaction was terminated by the addition of 0·06 ml glacial acetic acid. Blank runs were conducted in 50 mM citrate/phosphate buffer, pH 6·8 or in 50 mM citrate buffer, pH 4·5 under the same oscillatory conditions, but with no addition of α- or β-glucosidase sample.

Estimation of enzymatically liberated precarthamine content

The enzymatically liberated precarthamine content, after treating with or without supersonic oscillation, was determined by an indirect method (Fukushima *et al.*, 1990), where precarthamine was oxidised to carthamine and the amount of precarthamine was expressed in terms of carthamine (molecular mass = 910). Flower suspension was transferred to Teflon tubes and centrifuged at 4000 × *g* for 5 min. The supernatant was sucked out. Fresh water was added, the contents stirred with a glass bar and centrifuged again (4000 × *g*, 5 min). The washing process was repeated a further three times. To the combined washings (60 ml in total), 5 ml 10 μM $KMnO_4$ was added, stirred for few minutes, then 0·5 g Avicel cellulose was suspended and stirring continued for about 10 min on a magnetic stirrer. The suspension was subjected to centrifugation (4000 × *g*, 5 min) and the pellet washed three times with sufficient amounts of distilled water. The red Avicel was extracted with 100 ml 60% (v/v) acetone and the acetone extract used for the determination of carthamine content, which was followed by a spectrophotometric assay method as shown in one of our recent papers (Saito, 1993).

RESULTS AND DISCUSSION

The rate of precarthamine liberation increases hydrolytically after feeding the samples with both α- and β-glucosidase from yeast. The enzymatic action is pronounced by the process of supersonic oscillation. These are clear in the experiments conducted with or without a sono-sound oscillator. The data are comparable with each other in the following tables. Soundwave irradiation increases precarthamine yield by 19·9% in α-glucosidase-containing media (Table 1) and by 20·3% in β-glucosidase-containing mixtures on average (Table 2), indicating that sono-oscillatory treatment of reaction mixtures very efficiently liberates bound precarthamine. Perhaps, the machinery vibration promotes the hydrolytic release of precarthamine by stimulating α- or β-glucosidase activity during incubation for 10 min at 25 ± 1°C: all sonicated samples give rise to higher values of precarthamine dissociation compared with non-sono-oscillation of flower pastes. The most prominent effect of sono-sound oscillation is shown at 100 munit dosage of α- and β-glucosidase (rate of net increment: 45·9 and 36·1%, respectively, see Tables 1

Table 1. Precarthamine release by α-glucosidase in a reaction system treated with or without supersonic oscillation[a]

Enzyme fed (m units)	Sono-sound treatment	Precarthamine released (pmol precarthamine/ml/s)
1000	+[b]	3·33
	–[c]	3·30
100	+	3·61
	–	2·67
10	+	3·08
	–	3·02
1	+	3·24
	–	2·83
0·1	+	2·81
	–	2·20

[a]Blank run was 2·05 (pmol precarthamine/ml/s). For details of the treatment of reaction media with a supersonic oscillator, determination of released precarthamine contents or others, see the Materials and Methods section.
[b]Treated.
[c]Non-treated.

Table 2. Precarthamine release by β-glucosidase in a reaction system treated with or without supersonic oscillation[a]

Enzyme fed (m units)	Sono-sound treatment	Precarthamine release (pmol precarthamine/ml/s)
1000	+[b]	2·42
	–[c]	2·17
100	+	2·92
	–	2·30
10	+	2·89
	–	2·64
1	+	2·89
	–	2·61
0·1	+	3·17
	–	2·83

[a]Blank run was 1·72 (pmol precarthamine/ml/s). For details of the treatment of reaction media with a supersonic oscillator, determination of released precarthamine contents or others, see the Materials and Methods section.
[b]Treated.
[c]Non-treated.

and 2). Tests with other units dosages also yield sono-sound effects at lesser levels, showing varied increment ranges of 1·4–29·8% in α-glucosidase or of 14·5–19·8% in β-glucosidase. As a whole, β-glucosidase seems to yield constantly higher effects on precarthamine release under sono-sound regulated vibration, than α-glucosidase. The apparent differences in sono-chemical effect may results from the absolute configuration of precarthamine at the location of binding in cellular compartments. We suppose that β-configuration plays a leading role in the interaction between precarthamine and cellular components. On the basis of the present data, a possible co-existence of the α-configuration in the chemical bonding can not be ruled out, because considerable amounts of precarthamine are recovered by α-glucosidase catalysis, which is enhanced through the supersonication process. Considering that cellular components are of various complexities, precarthamine is possibly bound by various mechanisms, in which β-configurations, and α-configurations are involved.

In one of our preceding papers, we have shown that peroxidase-mediated carthamine decoloration is facilitated by supersonic radiation (Saito *et al.*, 1991). Supporting the previous finding, affirmative results have been provided by the current work: hydrolytic enzyme processes are also promoted by intense sonication. General knowledge about sono-chemical effects has already been outlined in a recent observation (Saito & Miyakawa, 1994). No clear cut explanation of the mechanism by which hydrolytic enzyme catalysis is enhanced by supersonic irradiation can be extracted from this study. The fact remains that we are now able to increase precarthamine production by exposing flower pastes to soundwave sonication. The orange-yellow dye thus prepared is used as a colorant and this may lead to greater practical utility of the *Carthamus* dye. The quinoidal chalcone glycoside is soluble in water and other aqueous solvents, showing a very fine yellow tincture, which prompts us to apply it as a new colorant for processed foods: it dyes foods yellow, orange–yellow, orange and/or reddish orange. Though no test for the toxicity/safety of the pigment has yet been done, it is no doubt edible, because its oxidation product, carthamine has been shown to be harmless in mice tests (Saito & Fukushima, 1991) and is now used as a food colorant in Japan. This new protocol is very simple and easy to execute. Hence, it is applicable to the dye production on a semi-industrial scale.

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