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A new technique for the chemical processing of reddened florets from dyer's saffron capitula

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A dilute solution of H_2O_2 expedites the reddening process of orange–yellow florets from dyer's saffron capitula. The catalysis proceeds stoichiometrically: both incubation time and H_2O_2 composition become crucial factors for the batho-shift reaction, and a value of 0·184 μM H_2O_2 is calculated from the double-reciprocal plots of H_2O_2 concentration vs velocity of carthamin production at pH 5·0. Inhibition tests, with various antioxidants, indicate that the batho-chromic colour shift is controlled by electron-chain reactions. The results are evaluated for practical application to prepare the raw material of carthamin dye.

ABBREVIATIONS

BAW	*n*-Butanol/acetic acid/water (3 : 1 : 1, v/v)
HOAc	Acetic acid/water (15 : 85, v/v)
PW	Phenol saturated with water
TLC	Thin-layer chromatography
UV	Ultraviolet light
VIS	Visible light.

INTRODUCTION

The reddened florets of dyer's saffron are indispensable for the preparation of carthamin dye. To prepare the raw material, three methods are practicable: (1) the classical procedure, which uses a native enzyme process (Saito, 1989), (2) a patented method using a glucose oxidase sample (Wada & Ota, 1986) and (3) a chemical technique originally devised by Saito (1991*a*). The first procedure is very difficult, as mentioned elsewhere (Saito, 1991*a*). The second seems to be somewhat disadvantageous, because the enzymic process is readily affected by external conditions, in particular by temperature, pH, O_2 content and microbial contamination. The third, which uses $KMnO_4$ as a red-colour initiator, is obviously superior to the other two in all respects, unless manganate ions are to be analysed subsequently. The significant aspects of permanganate ions have been described in our previous communications (Saito, 1991*b*; Saito & Katsukura, 1992).

To improve the third method, we have searched for more reliable batho-shift inducers. This paper will communicate another approach to the preparation of reddened florets from dyer's saffron, chemically, by a new technique which avoids noxious metal cations.

MATERIALS AND METHODS

Materials

H_2O_2, L-(+)-ascorbic acid, hydroquinone and α-tocopherol were purchased from Wako Pure Chemical (Osaka, Japan). D-Isoascorbic acid was obtained from Nakarai Kagaku Yakuhin (Kyoto, Japan). The other chemicals and reagents used were supplied from several commercial sources. Carthamin used as a standard specimen was prepared by the chemical technique of Saito *et al.* (1992). Silica-gel TLC plates were obtained from Merck (Darmstadt, Germany).

Fresh florets of dyer's saffron were collected from our experimental field on 22–27 August 1992, and used immediately for the scheduled experiments.

Administration of H_2O_2 to florets

Fresh florets (1 g each) were ground for 5 min in a mortar and pestle with 20 ml of 50 mM citrate buffer, pH 5·0, containing 10·9 μM H_2O_2, into small pieces, and the paste was left in the air at room temperature (23 ± 2°C) for 25 min.

Determination of H_2O_2 efficacy for the floret reddening

The reddened florets from the above process were subjected to extraction and partial purification of the

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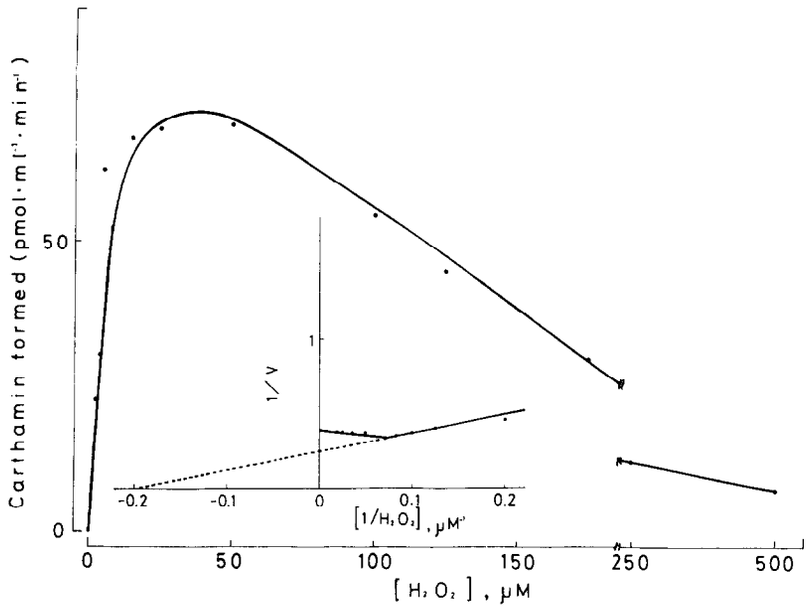

Fig. 1. Effect of H_2O_2 concentration on floret reddening. The inset shows the double-reciprocal plots: the value of $1/v$ is plotted on the ordinate in units of pmol/ml/min. Data were averaged from two separate experiments. For further details, see the Materials and Methods section.

resulting pigment. The method standardized by Saito *et al.* (1992) was followed. The pigment thus prepared was used for the spectrophotometric assay and its content calculated with a calibration curve. For the assay process, a Hitachi spectrophotometer, model U-1100, was used with 60% (v/v) acetone as reference. The data shown in the Table and Figures were averaged from two separate experiments.

Inhibitor tests

To characterize the reaction mode induced by H_2O_2 addition, four different inhibitors, hydroquinone, L-(+)-ascorbic acid, D-isoascorbic acid and α-tocopherol, all of which have generally been used as typical electron-chain breakers, were applied each at the 10·9 μM level. Fresh florets (1 g each) were ground in 20 ml of H_2O_2 (10·9 μM) solution in the presence of the inhibitors for 5 min at 23 ± 2°C and the paste was left for a successive 25 min in the air. The effect of the inhibitors on the rate of the H_2O_2-induced red colour development was estimated spectrophotometrically as described above.

Table 1. Effect of antioxidants[a] on H_2O_2-dependent floret reddening

Antioxidant	Carthamin formed[b] (pmol carthamin/ml/min)	Inhibition (% of control)
Control	320·6	100
L-(+)-Ascorbic acid	215·5	32·8
D-Isoascorbic acid	215·5	32·8
Hydroquinone	238·0	25·8
α-Tocopherol	204·9	36·0

[a] 10·9 μM antioxidant and 10·9 μM H_2O_2 were used in this study.
[b] The data were averaged from two separate experiments.

Tentative identification of reaction product

The partially purified pigment was identified by two different methods, chromatography and spectrophotometry. The former was carried out in three developing solvents on silica TLC plates in (A) BAW, (B) HOAc and (C) PW. The latter was performed with a Hitachi model U-3210 spectrophotometer with 60% (v/v) acetone as the solvent or the reference.

RESULTS AND DISCUSSION

H_2O_2 can induce red coloration in triturated orange–yellow floret pieces when added at a millimolar concentration. The resulting pigment was coincident with a marker carthamin on silica TLC plates developed in solvents (A), (B) and (C). The R_f values observed were (H_2O_2-induced product/authentic cathamin): 0·49/0·47, 0·59/0·58 and 0·27/0·25. The UV/VIS data of H_2O_2-induced pigment and a carthamin standard indicated that both spectra are basically identical: H_2O_2–pigment/authentic carthamin (nm), 291·3/280·4 (Band III); 340·6/339·9 (Band II); 521·3/521·3 (Band I). The apparent value of 0·184 μM was computed on the basis of the double-reciprocal plots of reaction velocity vs H_2O_2 concentration (see inset in Fig. 1). The H_2O_2-induced carthamin formation was affected by the pH values. The data obtained by screening the wide pH ranges from 3·0 to 7·0 suggested that the optimum activity is at pH 5·0 (Fig. 2). Values on both sides of this pH resulted in a serious decrease of the pigment production rate. The batho-chromic colour change in floret pieces was sensitive towards antioxidants. This can be shown by the tests with four antioxidants, all of which were used as typical electron-chain breakers. They reduce the red colour development with an inhibition rate

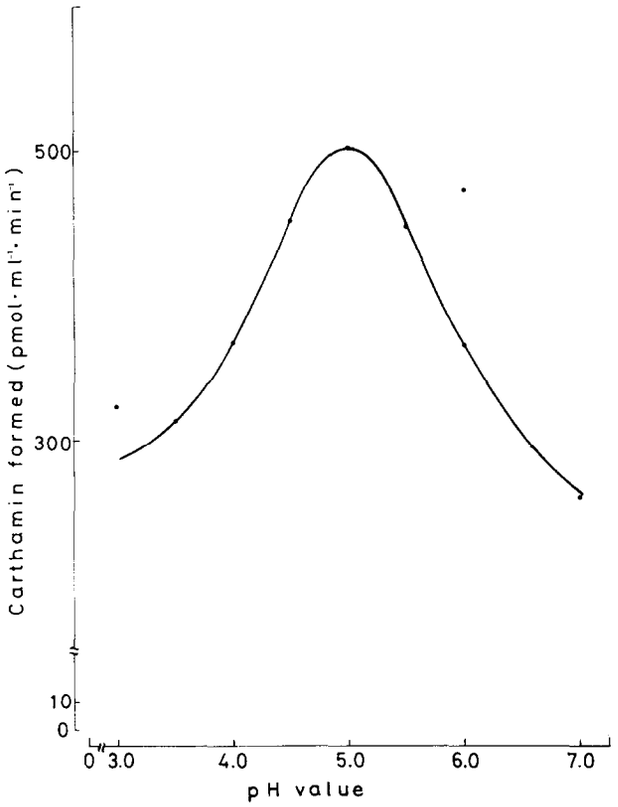

Fig. 2. Effect of pH on H_2O_2-induced floret reddening. Incubation was carried out in 50 mM citrate buffer as described in the Materials and Methods section, except that the pH values were varied as shown. Data were averaged from two different experiments.

ranging from 26 to 36% (Table 1), indicating the possibility that H_2O_2-induced colour shift is controlled by a series of electron-chain reactions. For steadying the yield of carthamin dye, one should take every possible care not to contaminate the raw materials with external dye inhibitors.

In one of our previous reports (Saito, 1991*a*), the permanganate method is shown to be excellent for processing reddened materials for the preparation of carthamin dye. This procedure is freely applicable to both fresh and dried florets of dyer's saffron without concern about the harvest time of starting materials or about microbial putrefaction as in performing the traditional method. The chemical technique is useful; however, the traces of manganate ions could become an important problem in the course of time, if they are left unrecovered.

The current findings will eliminate such problems. The H_2O_2 reagent contains no metal cations. It transforms readily to harmless O_2 and H_2O during the course of the oxidation reaction. The reagent is very effective: (1) the reddening process is completed speedily, (2) the procedure is simple, (3) it is freely applicable when required, if the fresh materials are stored under appropriate conditions and (4) it is inexpensive. The routinely applied method, a patented glucose oxidase method and a recently devised permanganate method (Saito, 1991*a*; Saito & Katsukura, 1992) will establish their places before long.

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