A Method for Preparation of Soluble Carthamin, Red Pigment from Safflower, Using Glycosyl Hesperidin

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Carthamin, the red pigment from *Carthamus tictorius* L. (safflower) is almost water-insoluble, so the compounds which solubilized it were screened from polysaccharides, flavonoids, and so on. When glycosyl hesperidin, which had the highest effect, was added to carthamin at the ratio of carthamin/glycosyl hesperidin=1/5 at pH 5.0, the content of soluble carthamin was about 14 times greater than that without its addition. The color tone of solubilized carthamin became more reddish, while the original one was yellowish red at pH 5.0 according to the Hunter Lab diagram system. On the basis of these results, carthamin solution could be prepared more effectively from the petals of safflower using glycosyl hesperidin instead of cellulose which is now used industrially.

Keywords: carthamin, safflower, *Carthamus tictorius* L., red pigment, glycosyl hesperidin, flavonoids

Carthamus tictorius L. is an annual herb of the compositae and is used in Chinese medicine for female-related diseases. The pigment in the flowers, called carthamin (Obara & Onodera, 1979; Takahashi *et al.*, 1982) has a vivid red color and is used in dyes, cosmetics and as a food colorant.

Carthamin has two characteristic properties, it is sparingly soluble in water but freely soluble in an alkali, and is specifically absorbed with cellulose. To make use of these properties, a commercial "carthamin preparation" was produced as described here. Dry petals of safflower were washed with water to remove the yellow pigments (safflor yellow A and B). Red pigment was then extracted with a Na₂CO₃ solution. Then, cellulose powder was added to this extract and neutralized. After washing with water, carthamin absorbed with cellulose was dried, and this was used as the commercial "carthamin preparation". However, as carthamin with cellulose is insoluble in water, it cannot be used in an aqueous system such as a beverage, dessert, and so on. Some investigations on this were made, but presently the only method in practice is the suspension of carthamin with cellulose. We therefore searched for a new technique to solubilize carthamin to allow broader application.

We earlier synthesized glycosyl hesperidin using transglycosylation reaction by cyclodextrin glucanotransferase from an alkalophilic *Bacillus* sp. A2-5a (Kometani *et al.*, 1994a). Glycosylation of hesperidin improved its solubility about 300 fold (Kometani *et al.*, 1994b). Furthermore, the addition of glycosyl hesperidin into canned mandarin orange and orange juice was found to prevent hesperidin crystal formation due to the solubility of water-insoluble hesperidin which was contained in the canned mandarin orange and orange juice (Terada *et al.*, 1995; Nishimura *et al.*, 1998). This phenomenon suggested that glycosyl hesperidin might solubilize flavonoids which had structures similar to it, as

described in the structural studies of flower pigments (Yoshida & Kondo, 1995).

Carthamin contains two glucosylated chalcone-structures in a molecule and glycosyl hesperidin has a glycosylated flavanone-structure in it. These structures of chalcone and flavanone are very similar (Fig. 1). Therefore, we tried to establish the new method for solubilizing carthamin using glycosyl hesperidin.

Materials and Methods

Materials Commercial "carthamin preparation", in which carthamin was absorbed in cellulose, was donated by San-Ei Gen F.F.I., Inc. (Osaka). Hesperidin dihydrochalcone and naringin dihydrochalcone were the gift of Nikken Fine Chemicals Co., Ltd. (Aichi). Glycosyl stevioside was from Nippon Seishi Industries (Tokyo). Hydroquinone- α -glucoside was produced as reported previously (Nishimura *et al.*, 1994).

Preparation of glycosyl hesperidin Glycosyl hesperidin was prepared as described (Kometani *et al.*, 1995). In short, the reaction mixture (20 l) containing 0.25% hesperidin, 5% soluble starch and 40,000 units of cyclodextrin glucanotransferase (1,4- α -D-glucan 4- α -D-(1,4- α -D-glucano)-transferase (cyclizing), EC 2.4.1.19, CGTase) from an alkalophilic *Bacillus* sp. A2-5a was incubated at 40°C and pH 10 for 16 h. The mixture was then put onto an Amberlite XAD-16 column. The resin was washed with H₂O to remove dextrin produced from soluble starch and glycosyl hesperidin was eluted with 50% ethanol. After concentration, the fraction containing glycosyl hesperidin (called Hsp-Gn in this study) was used for the solubilization of carthamin. Glycosyl hesperidin was further reacted with β -amylase from sweet potato and α -rhamnosidase from *Aspergillus niger*, and then the resultant reaction mixture was applied onto a second Amberlite XAD-16 column. The eluate with 50% ethanol was further purified with a Sephadex LH-20 column eluted with

Fig. 1. Structures of carthamin and glycosyl hesperidin.

50% ethanol. The fraction containing hesperidin mono and diglucoside at the molar ratio of 1:1 (called Hsp-G_{1&2} here) was collected and used to solubilize carthamin in this study, in order to make the results as clear as possible.

Assay for carthamin Carthamin was assayed with a HPLC system on an ODS column (Daisopak SP-120-10/20-ODS-B, Daiso Co., Ltd., Osaka), eluted with H₂O for the first 5 min and then with a gradient of 0–100% MeOH/H₂O (80:20, v/v) at a flow rate of 0.5 ml/min at 40°C, detecting absorbance at 530 nm. The concentration of carthamin in this study was calculated with a standard curve. It was also assayed with TLC on silica gel (Merck, Germany) developed with a solvent system of *n*-butanol/acetic acid/H₂O (4:1:2, v/v/v). Spots were detected visually as a vivid red color and also detected by spraying 50% (v/v) H₂SO₄ followed by heating at 130°C for 5 min.

Preparation of carthamin To prepare a pure carthamin sample, 10 g of a commercial “carthamin preparation” was extracted twice with 200 ml of 0.1 M Na₂CO₃ solution. After centrifugation, carthamin and cellulose were separated. Then, carthamin solution, adjusted to pH 6.0 with HCl, was applied onto a Sep-Pak(ODS) (Waters Chromatography, Millipore, MA) followed by washing with H₂O and eluting with 100% methanol. The eluate was concentrated *in vacuo* and finally freeze-dried. Fifty milligrams of carthamin was obtained as a powder.

Purity of carthamin was confirmed using the HPLC and TLC systems, that is, a single peak and a single spot, respectively.

Screening for the compounds which solubilize carthamin To screen the objective compounds, 20 mg of each compound tested was added to 1 ml of carthamin solution (1.23 mg/ml) which was solubilized with 0.1 M Na₂CO₃, and sufficiently mixed. The solution was adjusted to pH 5.0 by the addition of 1/10 volume (v/v) of 1 M sodium acetate buffer (pH 4.5), and after standing at 4°C for 16 h, it was centrifuged at 5000 *g* for 5 min. The absorbance at 530 nm of the resultant supernatant was measured.

Measurement of the solubility of carthamin Twelve, seventeen, and thirty five milligrams of carthamin was dis-

solved in 1 ml of 0.1 M Na₂CO₃. Glycosyl hesperidin was added to the solution at the ratio of carthamin/Hsp-G_{1&2}=5/1, 1/1, and 1/5 (w/w). One-tenth volume of 1 M sodium acetate buffer (pH 4.5) was added, and the pH of each solution became 5.0. As a control, 1/10 volume of H₂O was added to carthamin solution instead of the buffer. After standing at 4°C for 16 h, the supernatant was obtained by centrifugation (at 5000×*g* for 5 min). The concentration of soluble carthamin in the supernatant was measured by HPLC as described above.

Measurement of coloration of solubilized carthamin Color tone of solubilized carthamin was evaluated according to the three dimensional space system (Koda *et al.*, 1992), that is, the Hunter *Lab* diagram, which is expressed by *a* and *b* coordinated and *L* axis. In this system, *L* expressed lightness. Chroma which showed color brightness, hue which showed color tone, and difference in color (δE) was calculated by $(a^2 + b^2)^{1/2}$, $\tan^{-1}(b/a)$, and $(\delta L^2 + \delta a^2 + \delta b^2)^{1/2}$, respectively. The instrument used for measurement was a Multi Spectro Colour Meter MSC-IS-2DH (Suga Test Instruments Co., Ltd., Tokyo).

The starting solution contained 1.23 mg/ml of carthamin in 0.1 M Na₂CO₃. Hsp-G_{1&2} was added to the solution at the ratio of carthamin/Hsp-G_{1&2}=1/5 (w/w), and the pH was adjusted to 5.0. After standing at 4°C for 16 h, the coloration of the supernatant was measured.

Results

Screening for the compounds which solubilized carthamin As cellulose, β -1,4-glucan, was well known to absorb carthamin and used in a commercial “carthamin preparation”, we screened many saccharides including polysaccharides having β -1,4-linkages in their molecules. As shown in Fig. 2, β -cyclodextrin (β -CD), carboxymethylcellulose (CMC), pectin from lemon, mannan and fucoidan solubilized carthamin, but tamarind gum and chondroitin sulfate did not do so in this method, partly because of their insolubility in water. Cellulose was also not able to solubilize carthamin. Because cellulose was insoluble in water, carthamin-bound cellulose was precipitated. Most of the polysac-

charides tested had β -linkage like cellulose and were able to make carthamin water-soluble. However, as most of them tested made a solution viscous, they were thought not to be useful in practice.

As described in the Introduction, the effects of flavonoids including glycosyl hesperidin on the solubilization of carthamin were also investigated. As shown in Fig. 2, glycosyl hesperidin (Hsp-Gn and Hsp-G_{1&2}) and hesperidin dihydrochalcone were found to be the effective compounds. Many flavonoids such as neohesperidin dihydrochalcone, naringin dihydrochalcone, naringin, and neohesperidin could not solubilize carthamin much, and most of them precipitated because of their low solubility in water.

Fig. 3. Effects of the ratio of carthamin/Hsp-G_{1&2} on the solubilization of carthamin. (□), 12 mg/ml; (▨), 17 mg/ml; (■), 35 mg/ml of carthamin in the starting solution. Control represents the concentration of soluble carthamin without the addition of Hsp-G_{1&2}.

Thus, we selected glycosyl hesperidin to solubilize carthamin, because of its high solubility and having almost no taste, no flavor and no color.

Effects of glycosyl hesperidin on the solubility of carthamin Carthamin was dissolved in 0.1 M Na₂CO₃ in concentrations of 12 mg/ml, 17 mg/ml and 35 mg/ml, respectively. As a control, 1/10 volume of 1 M sodium acetate buffer (pH 4.5) was added to each carthamin solution and the pH of each solution was 5.0. The supernatant was obtained by centrifugation and contained 0.8 mg/ml of carthamin.

Hsp-G_{1&2} was added to carthamin solution at the ratio of carthamin/Hsp-G_{1&2}=5/1, 1/1, 1/5 (w/w) before pH adjustment. As shown in Fig. 3, according to the increase in the carthamin content in the starting solution and the decrease in the ratio of carthamin/Hsp-G_{1&2}, the concentration of soluble carthamin increased. Consequently, although the solubility of carthamin was not much improved by addition of Hsp-G_{1&2} at the ratio of 5/1, at the ratio of 1/1 and 1/5 the solubility was improved. When the ratio of carthamin/Hsp-G_{1&2} was 1/1 and 1/5 and carthamin content in the starting solution was 35

Fig. 4. Hunter Lab diagram of carthamin. (○), without Hsp-G_{1&2}; (●), with Hsp-G_{1&2}.

Fig. 2. Effects of various compounds on the solubilization of carthamin. 1, Hsp-Gn; 2, Hsp-G_{1&2}; 3, Glycosyl stevioside; 4, Hesperidin dihydrochalcone; 5, β -CD; 6, Carboxymethylcellulose; 7, Pectin from lemon; 8, Mannan; 9, Fucoidan; 10, Xanthan gum; 11, Polygalacturonate; 12, α -CD; 13, Soluble starch; 14, Xylan; 15, Neohesperidin dihydrochalcone; 16, Cellobiose; 17, Hydroquinone- α -glucoside; 18, Sodium alginate; 19, Naringin dihydrochalcone; 20, Phllozizin; 21, Arbutin; 22, Naringin; 23, No addition; 24, Chondroitin sulfate; 25, Neohesperidin; 26, Hesperidin; 27, Hesperetin; 28, Rutin; 29, Naringenin; 30, Glycyrrhizin; 31, Cellulose; 32, Tamarind gum.

mg/ml, the concentration of soluble carthamin was 4.9 and 11.7 mg/ml, respectively. Then, the ratio of carthamin solubility at pH 5.0, compared with the concentration of soluble carthamin without Hsp-G_{1&2} (None, in Fig. 3), was 5.5 and 13.5, respectively.

Characterization of solubilized carthamin The color of solubilized carthamin was evaluated according to the Hunter Lab diagram system. An aqueous solution of carthamin had a red to orangish-red color at pH 5.0, and an orange color at pH 7.0–10.5. Figure 4 shows the color of solubilized carthamin. In the range between pH 10.5 and pH 7.0, with or without the addition of Hsp-G_{1&2} at the ratio=1/5, the hue value, expressed color tone, increased according to the decrease in pH, indicating that the color changed to a reddish tone from yellowish red. At pH 5.0, without Hsp-G_{1&2}, the hue was not greatly different from that with Hsp-G_{1&2}, but the chroma value, expressed the brightness, decreased indicating that the red color had faded. On the other hand, with Hsp-G_{1&2}, chroma value increased and the color became brighter. Moreover, δE between the color of carthamin with Hsp-G_{1&2} and that without Hsp-G_{1&2} was 16.5 at pH 10.5, 15.8 at pH 9.0, 5.9 at pH 7.0, and 35.0 at pH 5.0, respectively. That showed the difference in color was greater at pH 5.0 than at other pH. The color tone changed to a more reddish one compared with those without Hsp-G_{1&2}.

Preparation of soluble carthamin from safflower petals Based on the results of this study, we attempted to prepare soluble carthamin from the petals of safflower using glycosyl hesperidin instead of cellulose which is used in industrial processes.

Fifty grams of dried safflower petals were extracted with

1000 ml of H₂O twice to remove the yellow pigments, safflor yellow A and B. After centrifugation, red precipitate containing carthamin was extracted with 500 ml of 0.1 M Na₂CO₃. Hsp-G_{1&2} or cellulose was added to the resultant red solution. The ratios of carthamin/Hsp-G_{1&2} in the extract were 1/1 and 1/5, respectively, and those of carthamin/cellulose were 1/5 and 1/8, respectively. Their pHs were adjusted to 5.8 with 1 M HCl. After being allowed to stand overnight, each carthamin solution was adjusted to pH 5.5 by the addition of 1/10 volume of 1 M sodium acetate buffer (pH 5.5), and the color tone measured according to the Hunter Lab diagram.

When cellulose was added to the carthamin solution, it absorbed the pigment specifically (Saito & Fukushima, 1988),

Fig. 6. Hunter Lab diagram of carthamin extracted from safflower. see legend of Fig. 5.

Fig. 5. Spectra of solubilized carthamin. 1, Carthamin solubilized by Hsp-G_{1&2} at the ratio of carthamin/Hsp-G_{1&2}=1/5 (referred to as Hsp-G_{1&2} (1/5)); 2, Carthamin solubilized by Hsp-G_{1&2} at the ratio of carthamin/Hsp-G_{1&2}=1/1 (Hsp-G_{1&2} (1/1)); 3, Control (without the addition of Hsp-G_{1&2} or cellulose); 4, Carthamin bound to cellulose at the ratio of carthamin/cellulose=1/5 (cellulose(1/5)); 5, Carthamin bound to cellulose at the ratio of carthamin/cellulose=1/8 (cellulose(1/8)).

Fig. 7. Coloration of carthamin extracted from the petals of safflower. 1, Carthamin solubilized by Hsp-G_{1&2} (the solution of Hsp-G_{1&2}(1/5)); 2, Carthamin bound to cellulose (the supernatant of cellulose(1/8)); 3, Carthamin precipitate (the precipitate of cellulose(1/8)).

and the complex of carthamin-cellulose was precipitated. The color of the resultant supernatant paled. As shown in Fig. 5, the absorption spectra decreased with the addition of cellulose, especially in the range between 480 nm and 550 nm, while those of each solution increased with the addition of Hsp-G_{1&2}. The absorbance at 530 nm of the solution containing Hsp-G_{1&2} at the ratio of carthamin/Hsp-G_{1&2}=1/5 was about 3.2 times greater than that of the control, and that of the solution containing cellulose at the ratio of carthamin/cellulose=1/8 was about 1.6 less than that of the control. The color of each carthamin solution was evaluated according to the Hunter Lab diagram, as shown in Fig. 6. The relationship of *L* value and chroma value indicated that the color tone became thicker, cellulose (1/8), cellulose (1/5), control, Hsp-G_{1&2} (1/1), and Hsp-G_{1&2} (1/5), in turn.

Figure 7 shows the color tone of solubilized carthamin by glycosyl hesperidin compared with that of carthamin bound to cellulose. The latter was easily precipitated, and the supernatant became pale and yellowish, as shown in No. 2. The resultant red precipitate is shown in No. 3. As Hsp-G_{1&2} solubilized carthamin (No. 1), the color tone of the solution became more reddish than that of carthamin bound to cellulose.

Discussion

Carthamin is an important red pigment because it has vivid tints of red and originates from natural products. Natural pigments have recently been noted from the viewpoint of safety. However, the use of carthamin has been limited because of its very low solubility in water. Thus, it is necessary to improve its solubility for wider application to food and cosmetics.

When we screened the compounds which solubilized carthamin, β -CD, pectin from lemon, mannan and fucoidan were selected among saccharides, and glycosyl hesperidin and hesperidin dihydrochalcone were done among flavonoids. Though many flavonoids could not solubilize carthamin much, flavonoids which had relatively higher solubility were able to do so. In our previous study, glycosyl hesperidin was found to prevent hesperidin crystal formation in an aqueous solution, canned mandarin orange and orange juice (Terada *et al.*, 1995; Nishimura *et al.*, 1998). The mechanism of the prevention was thought to be that glycosyl hesperidin might be incorporated into the growing hesperidin crystals because the structures were similar. With similar structures of carthamin and glycosyl hesperidin, the same mechanism would work. On the other hand, β -CD would solubilize carthamin by making an inclusion complex, which was supported by finding an inclusion complex of β -CD with hesperidin by analyzing with ¹H-NMR (Misaki, 1984). As the analysis revealed that β -CD formed a good inclusion complex with B-ring of hesperidin, it would also form a complex with phenol moiety of the carthamin molecule. Other saccharides seemed to be able to solubilize carthamin by specific binding with polysaccharides containing β -1,4 linkage like cellulose (Sawaki & Miyamoto, 1985; Shinagawa *et al.*, 1980; Shinagawa *et al.*, 1981).

Finally, we selected glycosyl hesperidin among these compounds as it was the most effective reagent. Glycosyl

hesperidin also has almost no taste and no flavor, and its solution is not as viscous as those containing polysaccharides.

Glycosyl hesperidin (Hsp-Gn) containing more than triglucosides was a little more effective than the mixture of hesperidin mono and diglucoside (Hsp-G_{1&2}), as shown in Fig. 2. This suggested that hesperidin tri-, tetra-, pentaglucoside and so on would also have the solubilizing activity like mono and diglucosides. As Hsp-Gn, Hsp-G_{1&2}, and hesperidin have the same aglycone, the difference between hesperetin and its glycosides is the number of saccharides such as glucose and rhamnose. Their carthamin-solubilizing activity decreased Hsp-Gn, Hsp-G_{1&2}, hesperidin, and hesperetin in turn; and the activities of Hsp-Gn and Hsp-G_{1&2} were much higher than those of hesperidin and hesperetin, which was proportional to their solubilities in water. Absorbance at 530 nm of the former was 8.36 and 7.95, and that of the latter was 1.22 and 1.11, respectively (Fig. 2). Furthermore, the chalcone-type structure had higher activity than the flavonol or flavanone-type as shown in hesperidin, neohesperidin, and naringin. Their aglycones, hesperetin and naringenin, had lower activity than did chalcones and flavonols or flavanones (Fig. 2).

From these results the carthamin-solubilizing activity of these flavonoids seemed to tend to be proportional to their solubility in water.

The relationship between carthamin content and glycosyl hesperidin content was then investigated. As shown in Fig. 3, the ratio of carthamin/Hsp-G_{1&2}=5/1 was not sufficient for the solubilization of carthamin, but the ratio of 1/1 and 1/5 achieved adequate solubilization.

In this study we solubilized water-insoluble carthamin and the color tone was much more reddish than the carthamin bound to cellulose. One question remains: was the reddish color tone was due to the increase in carthamin concentration or to the change in the color of carthamin itself? To answer this, solubilized carthamin solution (shown in Fig. 7 as No. 1) was diluted with H₂O to make the absorbance at 530 nm equivalent to that of carthamin solution without Hsp-G_{1&2} (No. 2 in Fig. 7). The coloration of each carthamin solution at pH 5.5 with or without Hsp-G_{1&2} was measured as described in Materials and Methods. The value of *L*, *a*, *b*, chroma and hue of carthamin solution without Hsp-G_{1&2} was 77.38, 11.63, 21.83, 24.73 and 0.92, respectively, and that of the solution with Hsp-G_{1&2} was 80.65, 17.64, 19.34, 26.18 and 1.20, respectively. δE of the latter solution against the former was 7.28. These values show that the solution with Hsp-G_{1&2} was a little more reddish than the solution without Hsp-G_{1&2}, but the difference was very small. Their spectra also showed the same result indicating solubilized carthamin had almost the same spectra curve (data not shown).

From these results, the color tone of solubilized carthamin was not greatly changed and the concentration of soluble carthamin increased with the addition of Hsp-G_{1&2}. We were able to make a solubilized carthamin solution without substantial change in the original color tone.

As a result of this study, we propose an improvement in the production of commercial "carthamin preparation": that glycosyl hesperidin be added to the alkaline extract from petals of safflower in place of cellulose. We could thus gain a

water-soluble carthamin preparation. Large-scale production is now in progress.

As our previous study revealed the effects of Hsp-G_{1&2} on stabilization of the pigmentation of natural color against ultraviolet ray irradiation (Kometani *et al.*, 1995), solubilized carthamin with Hsp-G_{1&2} may also be stable against this irradiation. With progress in the applied experiments, the red pigment may find broad use as a new food color.

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