Transglycosylation to Hesperidin by Cyclodextrin Glucanotransferase from an Alkalophilic Bacillus Species in Alkaline pH and Properties of Hesperidin Glycosides

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Cyclodextrin glucanotransferase [1,4-α-D-glucan 4-α-(1,4-glucan)-transferase, cyclizing; CGTase, EC 2.4.1.19] from an alkalophilic Bacillus species produced hesperidin monogluco- and a series of its oligogluco- and oligoglucosylates by the transglycosylation reaction with hesperidin as an acceptor and soluble starch as a donor. The formation of the oligosides was more effective at alkaline pHs than at neutral or acidic pHs, because of higher solubility of the acceptor.

The structure of the purified monoglucoside was identified as 4′-O-β-D-glucopyranosyl hesperidin by FAB-MS, α-, β-glucosidase and glucoamylase treatments, and methylation analysis.

The solubility of both hesperidin mono and diglucoside in water was about 300 times higher than that of hesperidin, and they were found to have a stabilizing effect on the yellow pigment crocin, from fruits of Gardenia jasminoides, against ultraviolet radiation.

Many biological activities of flavonoids have been described, inhibitory effects on mammalian enzymes, antiviral activity, and anticarcinogenic activity, etc. Furthermore, flavonoids are universally found either at the surface or in the epidermal cells of green leaves, and they seem to protect leaves from potentially damaging effects of atmospheric ultraviolet radiation. Such effects were confirmed by a report showing that Arabidopsis that had reduced synthesis of flavonoids was highly sensitive to the damaging effects of ultraviolet radiation. Therefore, flavonoids might be used as a protector against ultraviolet radiation. Although they have such useful properties, their use is limited, because of low solubility.

As it is known that most flavonoids are soluble in alkali, we tried to transglycosylate flavonoids in alkali and to solubilize them. For this purpose, we purified a new CGTase from an alkalophilic Bacillus sp. and investigated transglycosylation in alkaline pH.

Among these flavonoids, hesperidin has been known to have vitamin-like activity, which decreased capillary permeability and fragility. In addition, it can be produced from mandarin orange peel and seems to be one of the cheapest and safest flavonoids.

In this report, we described transglycosylation to hesperidin, characterization of its glycosides, and its use for stabilizing natural pigments against ultraviolet radiation.

Materials and Methods

Chemicals. Hesperidin and soluble starch were purchased from Sigma Chemical Co., Ltd. and E. Merck, respectively. Amberlite XAD-16 was purchased from Organo Co., Ltd.

Enzymes. CGTase was purified to a homogeneous state from an alkalophilic Bacillus sp. and the activity of the enzyme was assayed using soluble starch as a substrate by measuring the decrease in iodine-staining power as described previously. α-Glucosidase from yeast was purchased from Seikagaku Corporation, and one unit of the enzyme was defined as the amount of enzyme that formed 1 μmol of p-nitrophenol from p-nitrophenyl α-D-glucoside per min at 37°C and pH 6.8. β-Glucosidase from almonds was purchased from Sigma Chemical Co., Ltd. and one unit of the enzyme was defined as the amount of enzyme that formed 1 μmol of glucose from salicin per min at 37°C and pH 5. β-Amylase from sweet potato was from Sigma Chemical Co., Ltd. and one unit of the enzyme was defined as the amount of enzyme that formed 1 mg of maltose from starch in 3 min at 20°C and pH 4.8. Glucoamylase from Rhizopus sp. was purchased from Toyobo Co., Ltd. and one unit of the enzyme was defined as the amount of enzyme that formed 10 μmol of glucose in 30 min at 40°C and pH 4.5.

Transglycosylation. The standard reaction used in this study had a reaction mixture containing 0.1% (1.64 mm) hesperidin as an acceptor, 5% soluble starch as a donor, and 2 units of CGTase incubated at 40°C and pH 9 for 16 h. After the reaction, the mixture was boiled for 5 min and centrifuged at 5000 × g for 5 min to remove insoluble hesperidin. Transfer products in the supernatant were measured by HPLC on an ODS column (E. Merck) eluted with acetonitrile-phosphate buffer (pH 5.5; 20:80, v/v) at a flow rate of 0.5 ml/min at 40°C, detecting absorbance at 280 nm. The amount of hesperidin in the reaction mixture without enzyme was also measured by HPLC on an ODS as the soluble form. The amount of hesperidin glycosides was calculated by subtraction of the amount of residual hesperidin from that of the soluble one.

Purification of hesperidin glycosides. A reaction mixture (600 ml) containing 0.1% hesperidin, 5% soluble starch, and 200 units of CGTase was incubated at 40°C and pH 9. After this reaction for 16 h, it was put on an Amberlite XAD-16 column. The resin with adsorbed hesperidin glycosides was washed with H2O, and hesperidin glycosides was eluted with 50% ethanol. After this was concentrated in vacuo, the eluate was incubated with 10 units of β-amylase at 40°C for 1 h. The reaction mixture was boiled for 5 min and concentrated in vacuo. The concentrate was separated by FPLC on an ODS column eluted with 20% ethanol at a flow rate of 2 ml/min at room temperature, detecting absorbance at 280 nm. After the fractions containing glycosides were concentrated, they were put on a preparative TLC (E. Merck) on a silica gel developed with a solvent system of chloroform–methanol–H2O (65:35:10, v/v/v, lower layer). Four spots were detected by absorbance at 253 nm. One spot (glycoside D; Rf = 0.51) was found to be residual hesperidin by comparing it with the Rf of the authentic compound. Other spots seemed to be a series of hesperidin glycosides such as a hesperidin trigrucoside-like compound (glycoside A; Rf = 0.12), a diglucoside-like one (glycoside B; Rf = 0.19), and a monoglucoside-like one (glycoside C; Rf = 0.28). Then, each glycoside was scraped and extracted with H2O and obtained as a powder by lyophilization. Parities of these glycosides were confirmed by HPLC on an ODS described above, and by TLC, which was done with two solvent systems; chloroform–methanol–H2O (65:35:10, v/v/v, lower layer) and acetonitrile–H2O (80:20, v/v) detecting absorbance at 253 nm and spraying 50% (v/v) H2SO4 followed by heating at 130°C for 5 min.

FAB-MS analysis. FAB-MS data were obtained with a JMS-AXS300 system (JEOL) with a direct inlet system.
Enzymatic analysis. To analyze their structures, purified glycosides B and C were hydrolyzed by 1 unit of α-glucosidase, β-glucosidase, or glucoamylase at 40°C and pH 5 for 16 h. After hydrolysis, the released glucose was measured by the glucose oxidase method, and the released hesperitin was measured by HPLC on an ODS column, as described above.

Methylation analysis. The purified glycoside C (5 mg) was dried and dissolved in 0.25 ml of dimethyl sulfoxide with a small amount of sodium hydroxide and converted to the alkoxide with newly prepared methyl-3-ethylcarbanion at room temperature for 2 h with stirring. Then the mixture was mixed with 0.15 ml of methyl iodide for methylation and stirred at room temperature for 4 h. The methylated glycoside was extracted with chloroform repeatedly, and then the mixture was allowed to stand for 4 h. The methylated compounds were reduced with 13 mg of NaBH₄ at room temperature for 2 h, and acetylated with 0.5 ml of acetic anhydride-pyridine (1:1, v/v) at 100°C for 1 h. The mixture of partially methyalted alditol acetates was analyzed by gas chromatography (GC) with a TC-FFAP column (0.25 mm × 30 m; GL Sciences Inc., Tokyo) with helium gas as a carrier at the flow rate of 1 ml/min at 200°C. Standard partially methyalted alditol acetates were prepared from hesperitin and maltoyl-β-cyclodextrin by the same procedure.

Measurement of apparent solubility. Purified glycoside B and C were dissolved in H₂O at pH 6 and centrifuged at 5000 x g to remove insoluble compounds. The amount of glycoside in the supernatant of each glycoside B and C, which was diluted to a suitable concentration, was measured by HPLC on an ODS as described above. The apparent solubility was calculated with a standard curve.

Stabilization of the pigmentation of crocin. The solution containing 0.05% crocin and 0.01-0.1% hesperitin glycoside mixture (a mixture of hesperitin mono and diglucoside, 1:1, w/w) was left at 4°C and pH 4 under 12,000 lux. The solution containing 0.05% crocin and 0.1% saccharides such as glucose, maltose, soluble starch, and β-CD was also left under the same conditions. Control solution did not contain the hesperitin glycoside mixture. The absorbance at 442 nm of each solution was measured at 2, 4, 6, and 8 h. The residual pigmentation was expressed as the percentage of each absorbance measured, which was based on that of starting solution.

Results
Transglycosylation to hesperitin
To investigate the effects of pH on transglycosylation, a reaction mixture (1 ml) containing 0.1% (1.64 mm) hesperitin as an acceptor, 5% soluble starch as a donor, and 2 units of CGTase from an alkalophilic Bacillus sp. was incubated at 40°C for 16 h at various pH (using Britton–Robinson buffer, pH 3–13). As shown in Fig. 1, the amount of soluble hesperitin was greatly changed between pH 8 and 9. And the transfer products, a series of hesperitin glycosides, were also increased at pH 9 and 10, but gradually decreased above pH 11. The maximum amount of hesperitin glycosides was about 1.23 mm at pH 9.

To investigate the effects of the concentrations of acceptor and donor on transglycosylation, a reaction mixture (1 ml) containing 0.01–0.15% hesperitin, 0.5–5% soluble starch, and 2 units of CGTase was incubated at 40°C and pH 9 for 16 h. As shown in Fig. 2, increases of hesperitin and soluble starch concentrations increased the amounts of hesperitin glycosides.

To investigate the effects of enzyme activity and reaction time, a reaction mixture (1 ml) containing 0.1% hesperitin, 5% soluble starch and 0.5–10 units of CGTase was incubated at 40°C and pH 9 for 2–24 h. As shown in Fig. 3, as the reaction time was prolonged and the CGTase activity was increased, the amount of hesperitin glycosides was increased.

Purification of glycosides A, B, and C
When various glycosylated hesperidins were separated from a reaction mixture after elution from Amberlite

![Fig. 1](image1.png)

**Fig. 1.** Effects of pH on Transglycosylation to Hesperitin.
Standard reaction described in Materials and Methods was done in Britton–Robinson buffer (at various pHs, 3.13) (C), the amount of soluble hesperitin (mm)(●), the amount of hesperitin glycosides (mm) formed by CGTase reaction.

![Fig. 2](image2.png)

**Fig. 2.** Effects of Concentrations of Hesperitin and Soluble Starch on Transglycosylation.
Hesperitin at 0.05% (▲), 0.1% (●), and 0.5% (○) was used as an acceptor in the standard reaction described in Materials and Methods.

![Fig. 3](image3.png)

**Fig. 3.** Effects of CGTase Activity and Reaction Time on Transglycosylation.
CGTase at 0.5 u/ml (■), 1.0 u/ml (△), 2.0 u/ml (○), 4.0 u/ml (▲), and 10.0 u/ml (●) was used in the standard reaction described in Materials and Methods.
XAD-16, a series of transfer products were detected in the eluate by HPLC on an ODS as shown in Fig. 4-a. When the eluate was hydrolyzed by β-amylase, the glycosides A, B, C, and D were also detected by the same HPLC (Fig. 4-b), and the amounts of glycosides A and B were increased about 2.5 times that before β-amylase treatment. Glycosides C and D were not changed and other transfer products almost disappeared. Glycoside D was identified as residual (unreacted) hesperidin by comparison with the authentic one. On preparative TLC, three spots were detected by absorbance at 253 nm, which seemed to be a series of hesperidin glycosides such as hesperidin triglucoside, diglucoside, and monoglucoside, suggested from the results of hydrolysis by β-amylase and the well-known reaction pattern of CGTase. The spots were consistent with glycoside A, B, and C on an ODS (HPLC) described above. The yields were 14.2 mg, 15.4 mg, and 4.2 mg, for glycosides A, B, and C, respectively. The purities of these glycosides were confirmed by HPLC on an ODS and TLC described in Materials and Methods.

Table: Ratios of Partially Methylated Alditol Acetates from Glycoside C

<table>
<thead>
<tr>
<th>Alditol acetate</th>
<th>Glycoside C</th>
<th>Hesperidin</th>
<th>Maltosyl-β-cyclodextrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetra-O-methyl-glucitol acetate</td>
<td>1.00</td>
<td>—</td>
<td>1.00</td>
</tr>
<tr>
<td>2,3-Di-O-methyl-glucitol acetate</td>
<td>1.07</td>
<td>—</td>
<td>1.14</td>
</tr>
<tr>
<td>2,3,4-Tri-O-methyl-glucitol acetate</td>
<td>—</td>
<td>0.77</td>
<td>—</td>
</tr>
<tr>
<td>2,3,6-Tri-O-methyl-glucitol acetate</td>
<td>—</td>
<td>—</td>
<td>7.29</td>
</tr>
<tr>
<td>2,3,4-Tri-O-methyl-rhamnitol acetate</td>
<td>D'</td>
<td>D</td>
<td>—</td>
</tr>
</tbody>
</table>

| a | Numerical values represent the molar ratios of partially methylated alditol acetates from each sample, when the amount (moles) of 2,3,4,6-tetra-O-methyl-glucitol acetate is defined as 1.00. Moles were calculated by peak areas on GC and response factors. |
| b | The peak was detected and the molar ratio was not calculated. |

C was hesperidin α-D-glucopyranoside.

3. Methylation analysis. The purified glycoside C was tested by methylation analysis. GC of the alditol acetates of the methylated compounds of glycoside C showed the presence of 2,3-di-O-methyl-glucitol acetate, 2,3,4,6-tetra-O-methyl-glucitol acetate, and 2,3,4-tri-O-methyl-rhamnitol acetate as shown in Table. These results suggested that transferred glucose was attached at C-4 of the glucose moiety of hesperidin by a 1,4-linkage. From FAB-MS, enzymatic and methylation analyses, glycoside C was identified as 4′-α-D-glucopyranosyl hesperidin (Fig. 5).

Structures of glycosides A and B

To identify the structure of these glycosides, purified glycoside A was treated with 0.1 unit/ml of glucoamylase at 40°C and pH 5, and the products were analyzed by HPLC on an ODS described above at various intervals. As shown in Fig. 6, glycoside A was converted into glycosides B, C,
and D at 2 h and finally all into glycoside D. In addition, glycoside B was hydrolyzed with glucoamylase or α-glucosidase in the same manner as described in Enzymatic analysis of glycoside C section. The molar ratio of hesperidin to glucose was 1 : 1.82, 1 : 2.22, respectively, and it could not be hydrolyzed by β-glucosidase. These results suggested glycosides A and B were hesperidin tri and diglucoside, respectively.

**Apparent solubility of glycosides B and C**

Purified glycosides B (47 mg) and C (23 mg) was dissolved in 400 and 200 µl of H₂O. Their apparent solubilities were measured by HPLC on the ODS described above. Glycosides B and C could be dissolved in H₂O at least to 127 and 125 mM, respectively. Hesperidin as a control was treated in the same manner to give 0.45 mM as its solubility in H₂O.

**Stabilization of the pigment crocin by hesperidin glycosides**

Stabilization of the pigment crocin, a yellow color from fruits of *Gardenia jasminoides*, by hesperidin glycosides against ultraviolet light was investigated. As shown in Fig. 7, the residual pigmentation of the control solution decreased rapidly to about one-fourth that of the starting one by 4 h. It reached almost zero at 8 h. On the other hand, the residual pigmentation of the solutions containing 0.01, 0.05, and 0.1% hesperidin glycoside mixture were more stable than that of the control. They showed dose-dependency and the residual pigmentation of the solution containing 0.1% hesperidin glycosides was about 50% of that of starting one even at 8 h.

Comparing the effects of hesperidin glycosides with some saccharides such as glucose, maltose, soluble starch, and β-CD, the effect of β-CD was almost the same as that of 0.01% hesperidin glycoside mixture, and other saccharides had no effects.

**Discussion**

There are many reports on transglycosylation by CGTase, but few reports on transglycosylation at alkaline pH. We have described transglycosylation to various flavonoids at alkaline pHs because they were more soluble at alkaline pHs than at neutral or acidic pHs.

In this report, we tried to transfer glycosylation to hesperidin and described the glycosides formed.

Investigating the optimum conditions for transglycosylation to hesperidin, the amount of hesperidin glycosides was found to be much affected by the pH of the reaction mixture. The maximum amount of hesperidin glycosides was yielded at pH 9, and it was about 5 times greater than those at neutral or acidic pHs. This pH seems to be a critical point at which the solubility of hesperidin and the stability of CGTase from an alkalophilic *Bacillus* sp. were balanced, and the reaction proceeded effectively. In addition, the amount of hesperidin glycosides was proportional to that of soluble hesperidin up to pH 10. This also showed a high concentration of acceptor in a reaction mixture is desired in transglycosylation.

Furthermore, the amount of hesperidin glycosides formed increased, according to increases in the concentration of an acceptor and a donor, the activity of CGTase, and the reaction time. In conclusion, the reaction, in which 0.1% hesperidin, 5% soluble starch, and 2 units/ml of CGTase was incubated at 40°C and pH 9 for 16 h, was used as the standard conditions. On this condition, about 75% of the hesperidin yielded hesperidin glycosides.

Structures of hesperidin glycosides were thought to be as follows: Structural analysis of hesperidin monoglucoside showed that transferred glucose was linked to the C-4 glucose of the hesperidin molecule with α-1,4 linkage (Fig. 5). Hesperidin diglucoside yielded hesperidin and glucose and the molar ratio of hesperidin to glucose was about
1:2. Hesperidin triglucoside was hydrolyzed into hesperidin by way of producing di and monoglucoside (Fig. 6). Furthermore, as shown in Fig. 4, the treatment of the transfer products by β-amylase suggests that these glycosides had α-maltosyl moieties that were linked to each other with α-1,4 linkages.

From these results, transferred glucose moieties of hesperidin oligoglucosides were linked to the C-4 glucose of hesperidin sequentially and a series of hesperidin glycosides were produced by CGTase.

The solubility was greatly improved by transglycosylation, as in the case of rutin.14) As for hesperidin mono and diglucoside, each solubility at pH 6 was about 300 times greater than that of hesperidin.

The demand for natural pigments has increased, because of increasing consciousness of safety in food materials. However, one problem is the low stability of these pigments. Because diminishing the pigmentation of food extremely reduces its value as merchandise, natural pigments, such as the yellow pigment from the fruits of gardenia, must be improved in their stability for industrial uses.

Flavonoids are thought to be important in protection from the potentially damaging effects of atmospheric ultraviolet radiation, and hesperidin strongly absorbs ultraviolet light. Both hesperidin mono and diglucoside have almost the same absorption spectra as hesperidin (Fig. 8). When a pigment solution containing hesperidin glycosides was exposed to ultraviolet light, hesperidin glycosides seemed to stabilize the color of pigments by absorbing the ultraviolet light (Fig. 7). In addition, they do not have strong spectra in visible light (Fig. 8). Furthermore, they have almost no specific flavor and taste, so they might be used as stabilizers of pigments in food against ultraviolet light.

As further applications of hesperidin glycosides in addition to the stabilization of pigments and vitamins etc. against ultraviolet radiation, there are anti-hypertensive effects on blood pressure, so solubilized so-called vitamin P may be expected. Precise studies in this field are now needed.

References