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Studies on Biosynthesis of Novel Glycosides and Their Utilization

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Takashi Kometani

CONTENTS

INTRODUCTION

There are many compounds having biological and physiological activities in nature. However, many of them have disadvantages such as low stability, low solubility and unpleasant palatability. Furthermore, they exist a very small amount in nature, e.g. biologically active components in Chinese medicines. Therefore, the utilization of these compounds is limited in food processing.

Recently, most people have been paying much attention to their health, and requiring foods for keeping their health. Just at that time, physiologically functional foods,[1] which designed to provide specific health benefits, were proposed and some of them are already on the market in Japan including hypoallergenic rice[2] and low-phosphate milk.[3] In the situation where the consumers' interest and demand in food is natural and healthy, active substances originated from nature are enthusiastically needed in food industry. But for the reasons described above, there are very few compounds that can be used in food manufacturing. To utilize such active ones, especially as a food ingredient, it is necessary to improve their disadvantages, and to obtain a large amount of them easily and cheaply.

Glycosylation is considered to be a very useful method for improvement of the chemical properties and physiological functions of biologically and pharmacologically useful compounds. As in the case of salicylic acid,[4] because glycosylation improved both solubility and stability without reducing its biological activities, the oral administration of the

glucoside was found to exhibit a quicker and longer analgesic effect than does the aglycone (salicylic acid). Furthermore, the glucoside did not generate gastric ulcer, which is frequently induced by the aglycone. In the case of stevioside (13-*O*-β-sophorosyl-19-*O*-β-D-glucosyl steviol),[5] glycosylation revealed the remarkably improvement in both the intensity and character of sweetness of the products, which were mono and diglucosylated at 13-*O*-β-sophorosyl moiety. Glycosylated stevioside has been used as a new sweetener in food such as ice cream and soft drinks in Japan.

The aim of this study was to improve biologically active compounds by glycosylation, and to apply the glycosides formed to food manufacturing as novel ingredients.

The substrates to be glycosylated and the methods to glycosylate them were summarized in Table. These biologically active compounds were structurally classified into three kinds of groups, saccharides, glycosides, and aglycones. And aglycones were further classified into those having phenolic OH groups and those having alcoholic OH groups. Among these substrates, there were many reports on glycosylation to saccharides,[6] and studies on glycosylation to aglycones having alcoholic OH groups have been progressed using glycosidases[7,8] and amylase.[9] On the other hand, glycosylation to both glycosides and aglycones having phenolic OH groups have not been investigated sufficiently. Therefore, the author focused on glycosylation to these latter two kinds of compounds.

Table. Summary of Transglycosylation.

Substrates	Groups to be transglycosylated	Transglycosylated products	Methods
Saccharides	Saccharides	Glycosyl sucrose	
Glycosides	Saccharides	**Hesperidin glycosides**	CGTase (Alkalophilic *Bacillus* sp.)
		Salicin glycosides	CGTase (*Bacillus macerans*)
Aglycones	Phenolic OH groups	**Vanillin glucoside**	*Coffea arabica* Cells
		Capsaicin glucoside	*Coffea arabica* Cells
		3,4-Dimethoxyphenol glucoside	*Coffea arabica* Cells
	Alcoholic OH groups	Kojic acid glucoside	

*; The glycosides formed in this study were shown by underlining.

There are thought to be two methods for glycosylation, chemical and biochemical ones. The author choosed biochemical one, and the reasons were following; Chemical synthesis of glycosides is a multiple-step reaction consisting acetylation, glycosylation, and deacetylation, and it results in the production of a mixture of glycosides with α- and β-configuration. And it always accompanys with some byproducts. On the other hand, biochemical synthesis of glycosides is one-step and position-specific reaction in a mild condition. Therefore, biochemical methods are thought to be suitable to the purpose of this study.

As for the improvement of glycosides by transglycosylation, the author conducted to improve the properties of flavonoids. Recently, flavonoids were reported many biological activities, such as anticarcinogenic activity[10] and the effects of DNA protection from ultraviolet radiation damage.[11] As flavonoids

are widely distributed in plants, they became one of the most interesting substances in the plant components. But their use was limited because of their low solubility. As the method to transglycosylate them, cyclodextrin glucanotransferase (CGTase) was used, which had known to have a strong transglycosylating activity aud had possibility to transglycosylate various glycosides.[12] In general, higher concentration of substrate is desired in a transglycosylation reaction. In this study, transglycosylation in alkaline pH where most of flavonoids dissolved more than in neutral and acidic pH was tried for the first time.

As for glycosylation of aglycones having phenolic OH groups, one such enzyme is UDP-sugar glycosyltransferase isolated from plants.[13] The glycosides produced have the β-linkage at C-1 of the glucose moiety, which is the same structure as those existing in nature. However, UDP-sugar is very expensive and now there are only a few reports on microbial enzymes that could glycosylate them.[14,15] Therefore, the author conducted to glycosylate compounds with phenolic OH groups using cultured plant cells, which had a strong activity of glycosyltransferase and cultured on an inexpensive medium.

This thesis deals with biosynthesis of novel glycosides using microbial enzymes and cultured plant cells, and their utilization for food manufacturing.

In Chapter I, the author described the improvement of flavonoids by transglycosylation with CGTase from an alkalophilic *Bacillus* species in an alkaline pH range, and revealed some properties of glycosylated flavonoids. And the utilization of the glycosylated flavonoids for stabilization of the

natural pigments was investigated. In addition, the author also described synthesis of polyglucosylated glycosides, which precipitated spontaneously like amylose, by CGTase from *B. macerans*, and developed a new method for simple preparation of glycosides from crude extract in order to obtain a large amount of useful glycosides easily.

In Chapter II, the author described glucosylation of aglycones having phenolic OH groups, such as vanillin and capsaicin by cell suspension-cultures of *Coffea arabica*, and some properties of the glucosides formed. And synthesis of 3,4-dimethoxyphenol glucoside, which inhibits the absorption of glucose from small intestine, was also investigated by the same cell cultures in order to obtain a large amount of the glucoside.

CHAPTER I.

Synthesis of Glycosides by Cyclodextrin Glucanotransferase

Section 1. Purification and Characterization of Cyclodextrin Glucanotransferase from an Alkalophilic *Bacillus* Species and Transglycosylation at Alkaline pHs

Introduction

Many flavonoids such as hesperidin and rutin were known as vitamin P, which had strong hypotensive effects.[16] Some of them can be converted into the extremely sweet dehydrochalcone derivatives by hydrogenation.[17] Furthermore, flavonoids are universally present either at the surface or in the epidermal cells of green leaves, and they seem to protect leaves from the potentially damaging effects of atmospheric ultraviolet radiation and to suppress lipid photoperoxidation by scavenging superoxide anion formed during peroxidation in chloroplast.[18] Therefore, they are expected to be used as a protector from ultraviolet radiation in human skin and as a natural antioxidant. However, since most flavonoids are water-insoluble or scarcely soluble in water, their uses were limited. As most flavonoids are soluble in alkaline solution, the author tried to transglycosylate them in alkaline pHs and solubilize them. For this purpose, the author purified a new 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* species and investigated transglycosylation at alkaline pH.

Materials and Methods

Enzymes. 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 mg of glucose in 30 min at 40 °C and pH 4.5.

Microorganisms. A small amount of soil was suspended in sterilized water and spread on modified II-medium agar plates[19] containing 1% K-100 (mixture of α- and β-cyclodextrin; 2:1, purity of cyclodextrins; 98%, Ensuiko Sugar Refining Co., Ltd.), 0.5% polypepton, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3, and 2% agar (pH 10.3). The plates were incubated at 37 °C for 24-72 h. The colonies isolated were inoculated into liquid-medium containing 1% soluble starch, 4% corn steep liquor, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% Na_2CO_3 (pH 9.6) and cultured at 33 °C for 72 h aerobically. Cyclizing and transglycosylating activities of the culture broth were measured. A strain named A2-5a was isolated from about 200 colonies.

Characterization of Bacillus sp. The microbiological characterization of the strain A2-5a was done according to "Bergey's Manual of Systematic Bacteriology."[20] The strain, A2-5a, was an aerobic, sporeforming, Gram-positive, motile, catalase-producing, and rod-shaped bacterium. It is clear that the bacterium should belong to the genus *Bacillus*.

Assay for starch-hydrolyzing activity (Iodine method). The starch-hydrolyzing activity was measured by Fuwa's method[21] with a slight modification. The reaction mixture (250 μl) containing 1.2% soluble starch (E. Merck) and 50 μl of the enzyme solution (suitably diluted with distilled water) was incubated at 40 °C for 10 min in Britton-Robinson buffer (at various pHs, described for each experiment). The reaction was stopped by the addition of 500 μl of 0.5 N acetic acid-0.5 N HCl (5:1, v/v). Then, 5 ml of 0.005% I_2 in 0.05% KI solution was added to 100 μl of this mixture, and the absorbance of the final mixture was measured at 660 nm. One unit of the enzyme (expressed as HU) was tentatively defined as the amount of enzyme that produced a 10% reduction in the intensity of blue color of amylose-iodine complex per minute under the conditions described. Unless stated otherwise, the activity of the enzyme was measured by the iodine method in this section.

Assay for cyclyzing activity. The reaction mixture (100 μl) containing 2.5% soluble starch and 0.2 HU of the enzyme was incubated at 40 °C for 60 min in Britton-Robinson buffer (at various pHs). The reaction was stopped by boiling for 5 min. The reaction mixtures were diluted 5 times with 50 mM acetate buffer (pH 4.8) and then incubated with 10 units of glucoamylase at 40 °C for 16 h. The cyclodextrin formed was measured with HPLC on an Aminex HPX-42A column (Bio-Rad Laboratories) eluted with H_2O at a flow rate of 0.4 ml/min at 80 °C detecting refractive index (RI). One unit of the enzyme was defined as the amount of enzyme that formed 1 μmol of cyclodextrin per minute under the standard condition.

Assay for transglycosylating (coupling) activity. The reaction mixture (250 μl) containing 0.1% salicin as an acceptor, 1.2% soluble starch as a donor, and 50 μl of the enzyme solution (suitably diluted with distilled water) was incubated at 40 °C for 10 min in Britton-Robinson buffer (at various pHs). The reaction was stopped by boiling for 5 min. Transfer products were treated with 10 units of glucoamylase at 40 °C for 16 h. The amount of salicin in the hydrolysate was measured by HPLC on an ODS column (E. Merck) eluted with methanol-H_2O (25:75, v/v) at a flow rate of 0.5 ml/min at 60 °C detecting absorbance at 270 nm. The residual amount of salicin before glucoamylase treatment was also measured by HPLC on an ODS as a blank. The efficiency of transglycosylation was expressed as the percentage of salicin that had been transglycosylated by the enzyme.

Transglycosylation to saccharides. The reaction mixture (100 μl) containing 250 mM saccharides (D-glucose, D-xylose, L-sorbose, and L-rhamnose) as an acceptor, 5% soluble starch as a donor, and 2 HU of the enzyme was incubated at 40 °C for 16 h at both pH 10 and pH 5. The reaction was stopped by boiling for 5 min. Transfer products were analyzed by TLC on a silica gel developed in the solvent system of ethyl acetate-acetic acid-H_2O (3:1:1, v/v/v) by the multiple ascending technique (three times). After drying, the transfer products were detected by spraying 50% (v/v) sulfuric acid, followed by heating at 130 °C for 5 min.

Transglycosylation to flavonoids. The reaction mixture (1 ml) containing 0.1% flavonoid (diosmin, hesperidin, naringin, and neohesperidin) as an acceptor was reacted in the same procedure of transglycosylation to saccharides. Transfer products were measured by HPLC on an ODS column 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 flavonoid in the mixture without enzyme was also measured by HPLC as soluble flavonoid. The amount of transglycosylated flavonoid was calculated by subtraction of the amount of residual flavonoid from that of soluble flavonoid.

Measurement of protein. Protein in the purification steps was measured by the Coomassie blue method of Bradford[22] using bovine plasma γ-globulin as a standard.

Measurement of molecular weight. The molecular weight of the CGTase was estimated by sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC on a TSK Gel G3000 SW column (Tohso Co., Ltd.) eluted with 0.1 M phosphate buffer (pH 6.9) containing 0.3 M NaCl at a flow rate of 0.7 ml/min at 40 °C detecting absorbance at 280 nm. The marker proteins used were yeast glutamate dehydrogenase (*Mr*, 290,000), pig heart lactate dehydrogenase (*Mr*, 142,000), yeast enolase (*Mr*, 67,000), yeast adenylate kinase (*Mr*, 32,000), and horse heart cytochrome c (*Mr*, 12,400) for gel filtration, and those used for SDS-PAGE are shown in the

legend to Fig. 1.

Results

Purification of CGTase

1. Preparation of the crude CGTase. The bacterium was cultivated aerobically at 33 °C for 72 h in a medium containing 1% soluble starch, 4% corn steep liquor, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 1% Na_2CO_3. After cultivation, crude enzyme solution was separated from cells by centrifugation and dialyzed against distilled water.

2. Starch adsorption. One and a half % corn starch and 18.6% ammonium sulfate were added to the dialyzate and stirred at 4 °C for 60 min. The corn starch that adsorbed the enzyme was filtered and washed with 22.8% ammonium sulfate solution. The enzyme was eluted from corn starch with 33 mM Na_2HPO_4 and ammonium sulfate was added to the eluate up to a final concentration of 57%. The precipitate was collected by centrifugation. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer.

3. Q-Sepharose chromatography. The dialyzate was put on a Q-Sepharose column equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After the column was washed with 20 mM Tris-HCl buffer (pH 7.5) containing 0.4 M NaCl, the enzyme was eluted with a linear gradient from 0.4 M to 1 M of NaCl. A single sharp peak having CGTase activity was obtained. Active fractions were collected and used as a purified CGTase. The purification procedure is summarized in Table.

Table. Purification of Cyclodextrin Glucanotransferase from an Alkalophilic *Bacillus* sp.

Steps	Total Volume (ml)	Total Units	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)
Crude Enzyme	1660	39000	1500	26	100
Starch Adsorption	510	31000	71	440	79
$(NH_4)_2SO_4$ ppt.	13	27000	52	520	69
Q-Sepharose	108	20000	34	590	51

Estimation of molecular weight

The purified CGTase gave a single band on SDS-PAGE as shown in Fig. 1, and a single peak on gel-filtration (HPLC) (data not shown). The molecular weight was estimated to be 80,000 (SDS-PAGE) and 70,000 (HPLC).

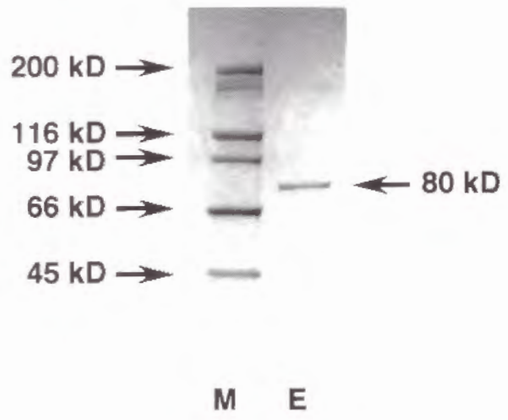


Fig. 1. SDS-PAGE of Purified CGTase from an Alkalophilic *Bacillus* sp.

E, purified CGTase; M, marker proteins; myosin (*Mr*, 200,000), *E. coli* β-galactosidase (*Mr*, 116,250), rabbit muscle phosphorylase *b* (*Mr*, 97,400), bovine serum albumin (*Mr*, 66,200), and hen egg white ovalbumin (*Mr*, 45,000).

Effects of pH on enzyme activity and stability

The three enzyme activities, cyclizing, transglycosylating (coupling), and starch-hydrolyzing activities, were measured at various pHs using Britton-Robinson buffer (at the range from pH 2 to 13). As shown in Fig. 2, the optimum pHs of the three enzyme activities were pH 5.5 at 40 °C, almost the same value. And each pH-activity curve was a single peak at the optimum pH with a broad shoulder in alkaline pHs; as for cyclizing and transglycosylating activities, the shoulder of the curve was from pH 7 to 10. The enzyme retained about 50% of its activity at pH 10 and 9, respectively; as for starch-hydrolyzing activity, it was from pH 7 to 11. The enzyme retained about 40% of its activity even at pH 11.

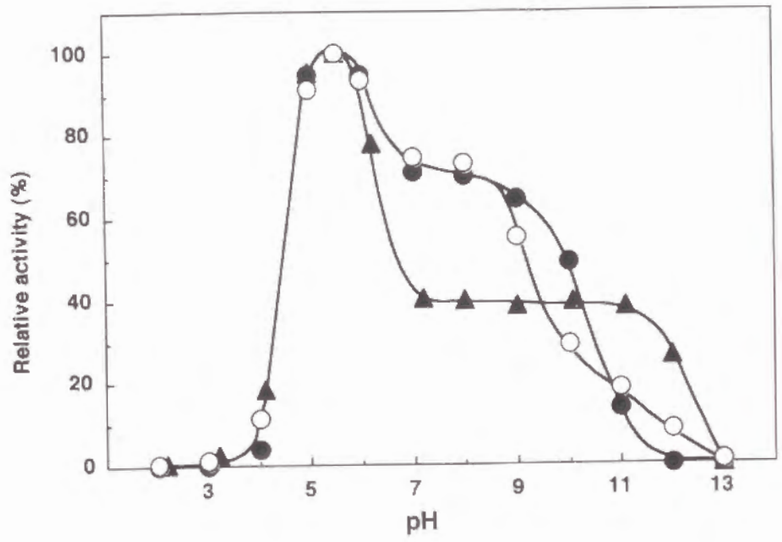


Fig. 2. Effects of pH on Activity.

The pH-activity curve of the purified enzyme was measured as described in Materials and Methods. (●), cyclizing activity; (○), transglycosylating (coupling) activity; (▲), starch-hydrolyzing activity.

The enzyme was incubated at 40 °C for 2 h at various pHs using the same buffer described above. The residual activity was measured at pH 5.5 by the iodine method. As shown in Fig. 3, the enzyme was stable at the range of pH 6-9 under the tested condition.

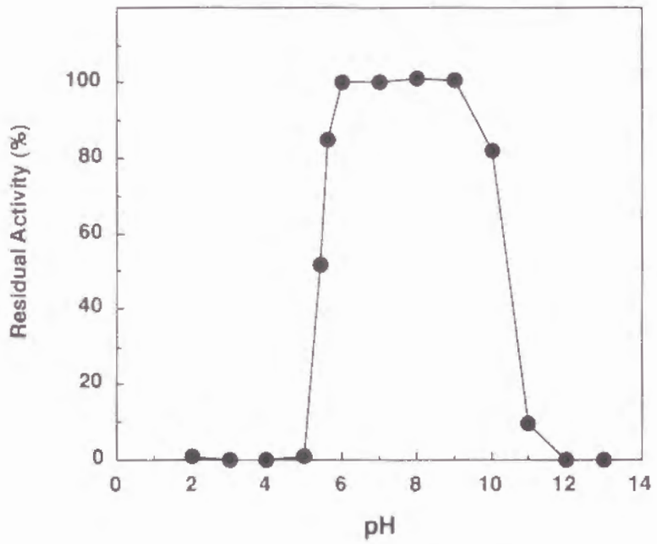


Fig. 3. Effects of pH on Stability.

Effects of temperature on enzyme activity and stability

The enzyme activity was also measured at various temperatures. The optimum temperature at pH 5.5 was 50-55 °C, as shown in Fig. 4.

The enzyme was incubated in Britton-Robinson buffer (pH 5.5, 7, and 10) at various temperatures for 10 min, and the residual activity was measured by the iodine method. As shown in Fig. 5, the enzyme was stable up to 60 °C at pH 7. In the presence of 1 mM $CaCl_2$, the enzyme was more stable and maintained 60% of its activity even at 70 °C.

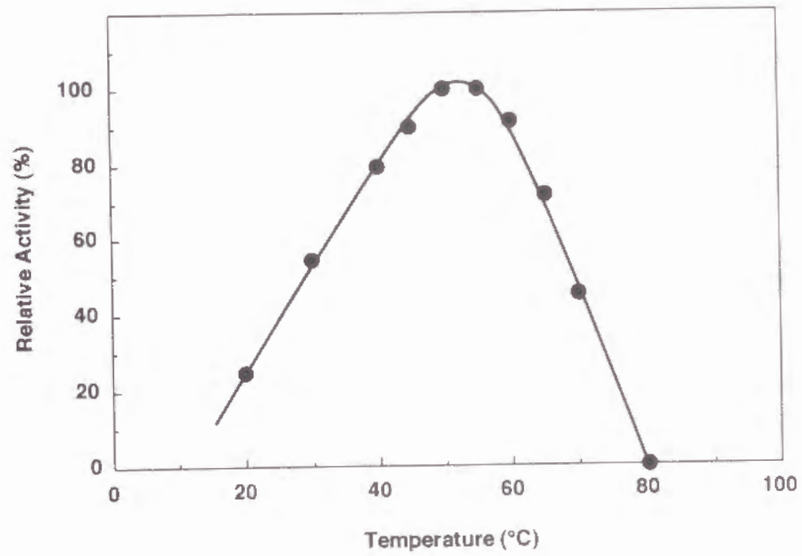


Fig. 4. Effects of Temperature on Activity.

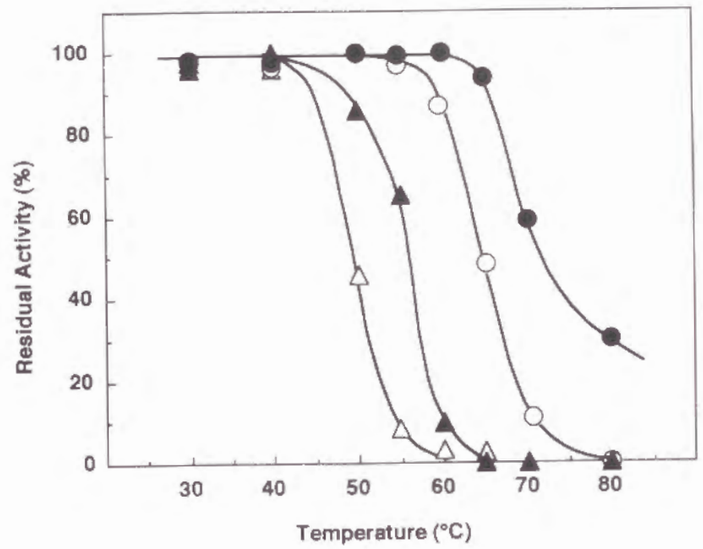


Fig. 5. Effects of Temperature on Stability.

The enzyme was assayed at pH 5.5 (△), at pH 10 (▲), and at pH 7 in the absence (○), or presence (●) of 1 mM $CaCl_2$.

Formation of cyclodextrins from starch

Formation of cyclodextrins from 0.5% soluble starch at 40 °C was measured by HPLC on an Aminex HPX-42A column. As shown in Fig. 6, during the first 20 min β-cyclodextrin was preferentially produced and the ratio of α-, β-, and γ-cyclodextrin was 0:7:1. After prolonged reaction, α- and γ-cyclodextrin were formed gradually, and β-cyclodextrin was decreased. As a result, the total amount of cyclodextrins formed was constant.

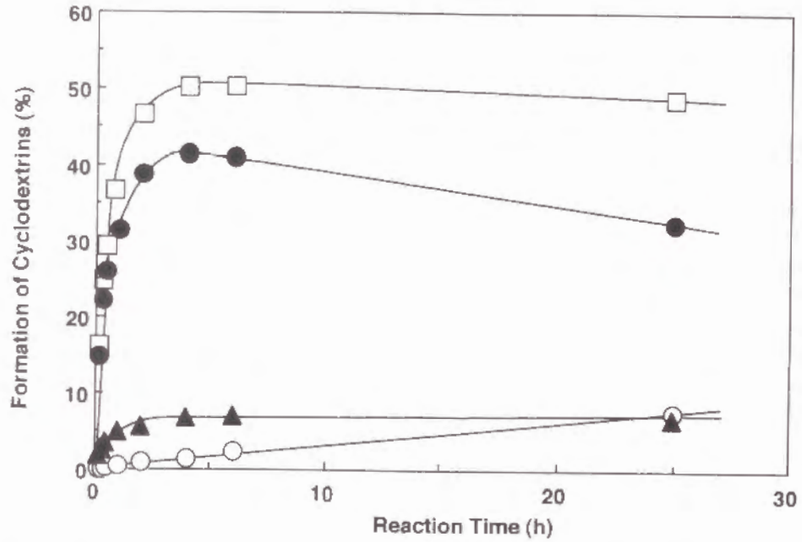


Fig. 6. Formation of α-, β-, and γ-Cyclodextrin from Starch.

(○), α-cyclodextrin; (●), β-cyclodextrin; (▲), γ-cyclodextrin; (□), total cyclodextrin.

Acceptor specificity in alkaline pH range

1. Transglycosylation to saccharides. As shown in Fig. 7, four saccharides tested were transglycosylated at both an alkaline and a neutral pH, and the amount of transfer products of glucose was almost same at both pHs. However, as for other saccharides, the amount of transfer products at pH 10

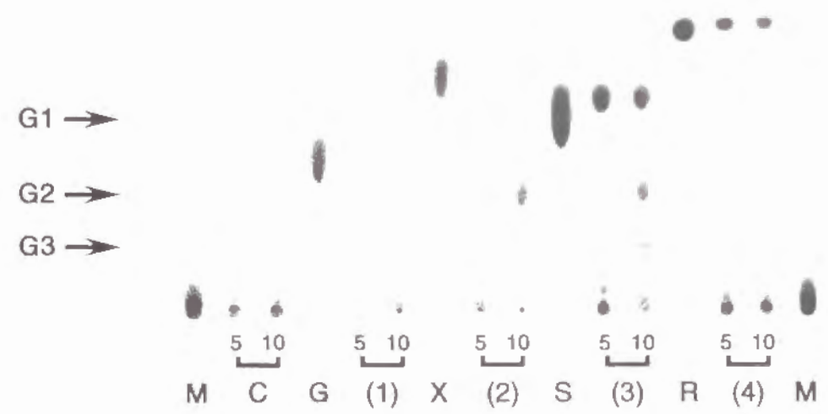


Fig. 7. TLC Patterns of Transglycosylation to Various Saccharides.

M, markers (maltooligosaccharides: Gl, glucose; G2, maltose; G3, maltotriose.); G, D-glucose; X, D-xylose; S, L-sorbose; R, L-rhamnose; C, control (reaction mixture without acceptor); (1), (2), (3), and (4), transfer products of D-glucose, D-xylose, L-sorbose, and L-rhamnose, respectively; 5 and 10, reaction was done at pH 5 and pH 10, respectively.

were greater than those at pH 5.

2. Transglycosylation to flavonoids. As shown in Fig. 8, comparing transglycosylation at pH 10 with that at pH 5, a great difference was found in diosmin. Diosmin was transglycosylated at pH 10 to give about 75% as the efficiency of transglycosylation. And that was not transglycosylated at pH 5, because it was insoluble at that pH. Hesperidin was also transglycosylated at pH 10, and the amount of hesperidin glycosides formed was about four times greater than that at pH 5. As for naringin and neohesperidin, these flavonoids were also transglycosylated at both pH 10 and 5 to give about 40% as the

efficiency of transglycosylation, but the amount of the glycosides formed at pH 10 was about two times greater than that at pH 5.

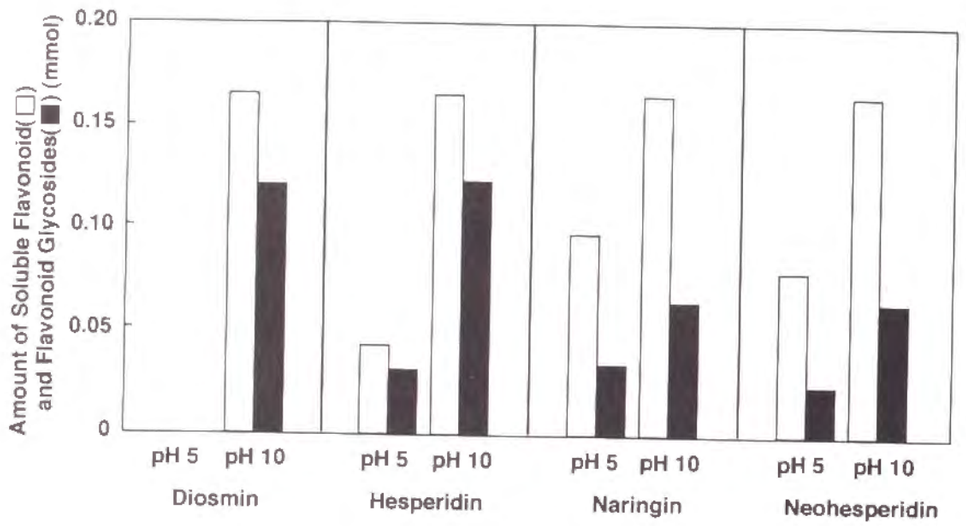


Fig. 8. Transglycosylation to Various Flavonoids.
(□), the amount of soluble flavonoid; (■), the amount of flavonoid glycosides formed.

Discussion

There have been many studies on transglycosylation by CGTase, i.e., transglycosylation to glucose,[23,24] sucrose,[25] stevioside,[5,26] rubusoside,[27] rutin,[28] and L-ascorbic acid.[29] But there are few reports about transglycosylation at alkaline pHs.

In this study, the author described research on transglycosylation at alkaline pHs for the first time. Transglycosylation to D-glucose by this new alkalophilic CGTase at alkaline pHs was not different from that at neutral pHs. However, for D-xylose, L-sorbose, and L-rhamnose, the amounts of transfer products in alkaline pHs tend to be greater than those in neutral pHs. Since the acceptor specificity of this enzyme to these saccharides seems to be the same as CGTase reported before,[12,30] it is interesting why transglycosylation occurs at a higher rate at alkaline pHs. Detail study is in progress. Great effects on transglycosylation in alkaline pH range were found on flavonoids, which were more soluble in alkaline pHs than in neutral or acidic pHs. Alkali-tolerant CGTase in this study could transglycosylate various flavonoids effectively in alkaline pHs. As described in the text, diosmin was soluble at pH 10, but insoluble at pH 5. It was able to transglycosylate only in alkaline pHs. As for other flavonoids, even though the efficiency of transglycosylation in alkaline pHs was as same as that in neutral pHs, the amount of products in the former condition was much higher than those in the latter condition, because they have higher solubility in alkaline pHs. In this experiment, 0.1% flavonoid (1.64 mM as hesperidin) was used as an acceptor. But practically, hesperidin was soluble to the concentration of about 2.62 mM at pH 10 (data not shown), and the

efficiency of transglycosylation was about 75%. From these results, the maximum amount of hesperidin glycosides formed was calculated to be 1.97 mM. Therefore, the amount of glycosides formed at pH 10 was calculated to be six times greater than that at pH 5.

Comparing acceptor specificity with four flavonoids, the author found that to transglycosylate rutinose (75% as the efficiency of transglycosylation in diosmin and hesperidin) was easier than to do neohesperidose (40% in naringin and neohesperidin).

There were reported some CGTases from an alkalophilic *Bacillus* sp.[31-34] When the pH-activity curve of each CGTase was measured by the iodine method. Horikoshi's had three peaks at an acid (pH 4.5), a neutral (pH 7.0) and an alkaline pH (pH 9.0)[31]; Nomoto's had two peaks at an acid (pH 5.0) and an alkaline pH (pH 9.0)[32]; Nakamura's had a single peak at pH 4.5-4.7 for acid-CGTase[33] and at pH 7.0 for neutral-CGTase.[34] CGTase in this study had a single peak (pH 5.5 as the optimum pH) with a broad shoulder in the alkaline pH range. There have been no reports of a pH-activity curve like that illustrated in Fig. 2. By the transglycosylation with an alkalophilic CGTase, the author could transglycosylate water-insoluble substances such as flavonoids; diosmin, hesperidin, naringin and neohesperidin, and solubilize them. These flavonoid glycosides might be used as physiologically functional materials for food and medicine with hypotensive activity and vitamin P activity, and might be developed for other useful applications, such as a protector against ultraviolet radiation and an antioxidant.

Summary

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* species was purified by starch adsorption and Q-Sepharose chromatography. The purified enzyme had cyclizing activity, transglycosylating (coupling) activity, and starch-hydrolyzing activity, and their pH-activity curves had a single peak (pH 5.5 as the optimum pH) with a broad shoulder at alkaline pHs.

Transglycosylation to various saccharides and flavonoids at alkaline pH was more effective than that at neutral pH. Among flavonoids, those containing rutinose (diosmin and hesperidin) were transglycosylated more effectively than those containing neohesperidose (naringin and neohesperidin).

Section 2. Transglycosylation to Hesperidin by Cyclodextrin Glucanotransferase from an Alkalophilic *Bacillus* Species at Alkaline pHs and Properties of Hesperidin Glycosides

Introduction

Many biological activities of flavonoids have been described, inhibitory effects on mammalian enzymes, antiviral activity, and anticarcinogenic activity, etc.[10] 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.[18] 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.[35] 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, the author tried to transglycosylate flavonoids in alkali and to solubilize them. For this purpose, the author purified a new CGTase from an alkalophilic *Bacillus* sp. and investigated transglycosylation at alkaline pHs (Chapter I, section 1).

Among these flavonoids, hesperidin has been considered to have vitamin-like activity, which decreased capillary permeability and fragility.[10] In addition, it can be produced from mandarin orange peel,[36,37] and seems to be one of the cheapest and safest flavonoids.

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

Materials and Methods

Enzymes. CGTase was purified to a homogeneous state from an alkalophilic *Bacillus* sp, and the activity of the enzyme was assayed using soluble starch (E. Merck) as a substrate by measuring the decrease in iodine-staining power as described in Chapter I, section 1. 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 μmole 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 μmole 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 mg 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 5,000 *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 1,200 units of CGTase was incubated at 40 °C and pH 9. After this reacted for 16 h, it was put on an Amberlite XAD-16 column (Organo Co., Ltd.). The resin with adsorbed hesperidin glycosides was washed with H_2O, 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-H_2O (65:35:10, v/v/v, lower layer). Four spots were detected by absorbance at 253 nm. One spot (glycoside D: R_f=0.51) was found to be residual hesperidin by comparing it with the R_f of the authentic one. Other spots seemed to be a series of hesperidin glycosides such as a hesperidin triglucoside-like compound (glycoside A; R_f=0.12), a diglucoside-like one (glycoside B: R_f=0.19), and a monoglucoside-like one (glycoside C; R_f=0.28). Then, each glycoside was scraped and extracted with H_2O, and obtained as a

powder by lyophilization. Purities of these glycosides were confirmed by HPLC on an ODS described above, and by TLC, which was done with two solvent systems; chloroform-methanol-H_2O (65:35:10, v/v/v, lower layer) and acetonitrile-H_2O (80:20, v/v) detecting absorbance at 253 nm and spraying 50% (v/v) H_2SO_4 followed by heating at 130 °C for 5 min.

FAB-MS analysis. FAB-MS data were obtained with a JMS-AX500 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,[38] and the released hesperidin was measured by HPLC on an ODS , 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 methylsulfinyl carbanion[39] 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 dried. The completely methylated glycoside was hydrolyzed with 90% (v/v) formic acid at 100 °C for 1 h, then with 1 N sulfuric acid at 100 °C for 4 h. The hydrolyzed compounds were reduced with

13 mg of $NaBH_4$ 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 methylated alditol acetates was analyzed by gas chromatography (GC) with a TC-FFAP column (0.25 mm × 30 m: GL Sciences Inc.) with helium gas as a carrier at the flow rate of 1 ml/min at 200 °C. Standard partially methylated alditol acetates were prepared from hesperidin and maltosyl-β-cyclodextrin by the same procedure.

Measurement of apparent solubility. Purified glycosides B and C were dissolved in H_2O at pH 6 and centrifuged at 5,000 *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 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% hesperidin glycoside mixture (a mixture of hesperidin 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 hesperidin 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 hesperidin

To investigate the effects of pH on transglycosylation, a reaction mixture (1 ml) containing 0.1% (1.64 mM) hesperidin 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 hesperidin was greatly changed between pH 8 and 9. And the transfer products, a series of hesperidin glycosides, were also increased at pH 9 and 10, but gradually decreased above pH 11. The maximum amount of hesperidin glycosides was about 1.23 mM at pH 9.

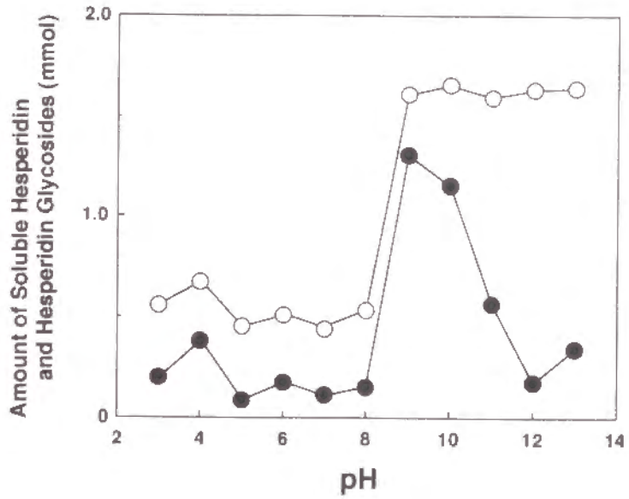


Fig. 1. Effects of pH on Transglycosylation to Hesperidin.

Standard reaction described in Materials and Methods was done in Britton-Robinson buffer (at various pHs, 3-12).

(○); the amount of soluble hesperidin (mmol)

(●); the amount of hesperidin glycosides formed (mmol)

To investigate the effects of the concentrations of acceptor and donor on transglycosylation, a reaction mixture (1 ml) containing 0.01-0.15% hesperidin, 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 hesperidin and soluble starch concentrations increased the amount of hesperidin glycosides.

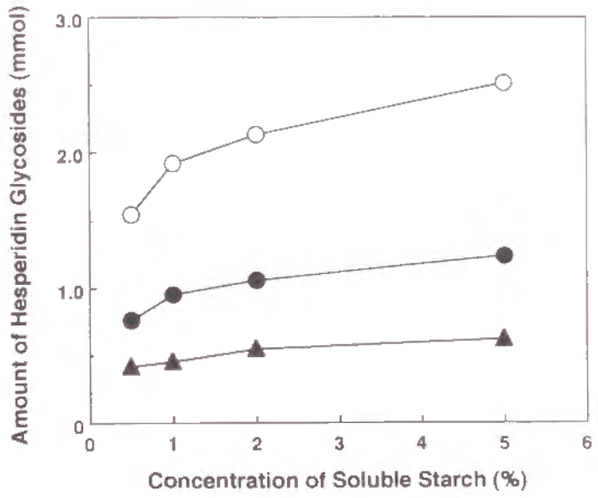


Fig. 2. Effects of Concentrations of Hesperidin and Soluble Starch on Transglysosylation.

Hesperidin at 0.05%; (▲), 0.1%; (●), and 0.5%; (○) was used as an acceptor in the standard reaction described in Materials and Methods.

To investigate the effects of enzyme activity and reaction time, a reaction mixture (1 ml) containing 0.1% hesperidin, 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 hesperidin glycosides was increased.

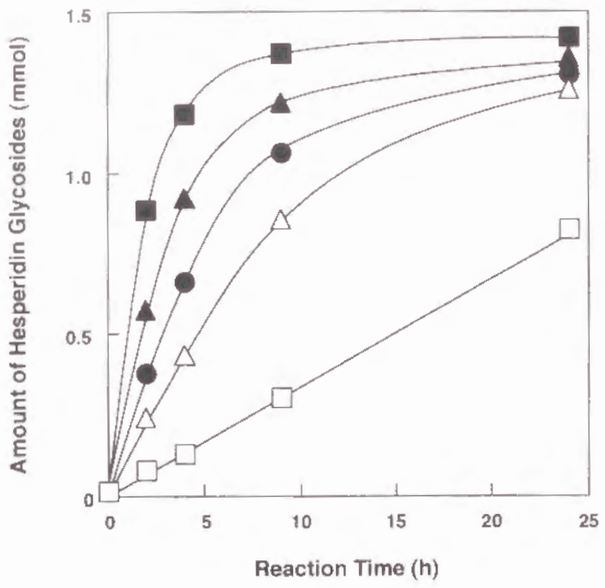


Fig. 3. Effects of CGTase Activity and Reaction Time on Transglycosylation.
CGTase at 0.5 u/ml; (□), 1.0 u/ml; (△), 2.0 u/ml; (●), 4.0 u/ml; (▲), and 10.0 u/ml; (■) was used in the standard reaction described in Materials and Methods.

Purification of glycosides A, B, and C

When various glycosylated hesperidins were separated from a reaction mixture after elution from Amberlite 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 did not changed and other transfer products almost disappeared. Glycoside D was identified as residual (unreacted) hesperidin by comparison with the authentic one. On a 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 glycosides 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.

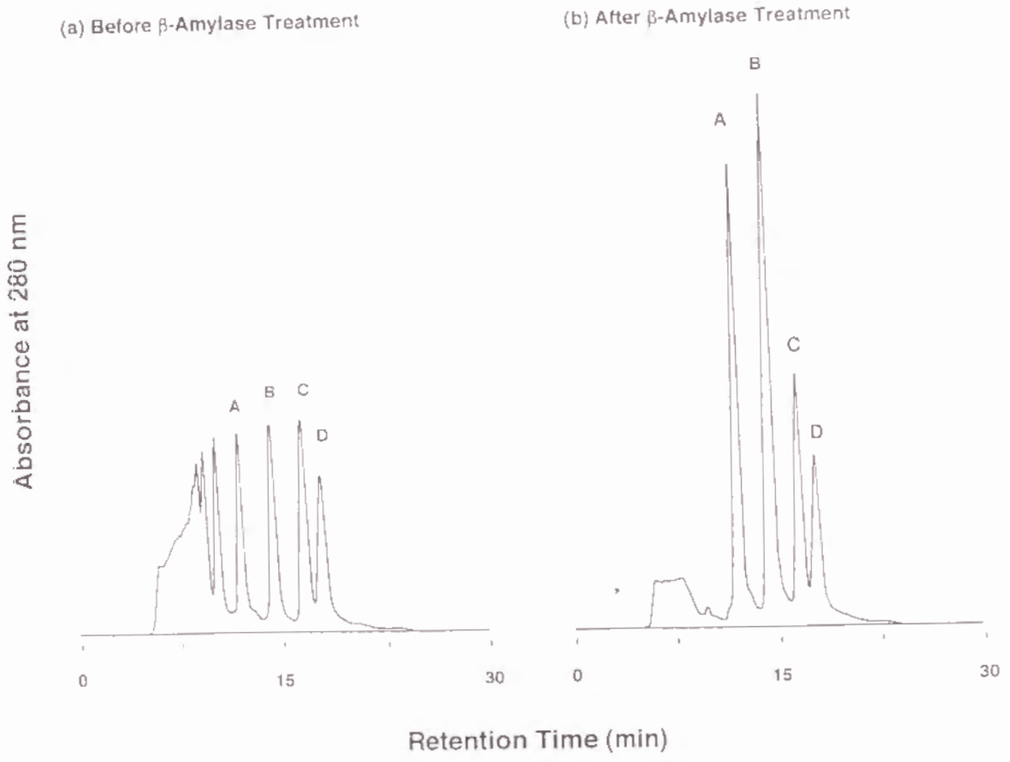


Fig. 4. HPLC(ODS) Patterns of Hesperidin Glycosides before and after β-Amylase Treatments.
In HPLC patterns (a); before and (b); after β-amylase treatments, glycosides A, B, C, and D represent hesperidin tri, di, and monoglucoside and hesperidin, respectively.

Structure of glycoside C

1. FAB-MS analysis.

The molecular mass of glycoside C was estimated to be 772 daltons by FAB-MS, which gave a molecular ion at m/z 773 ($[M + H]^+$).

2. Enzymatic analysis.

To investigate the structure of glycoside C, the purified product was treated with 1 unit each of α-glucosidase, β-glucosidase, and glucoamylase. After hydrolysis by glucoamylase, glycoside C (0.4 mg/ml) yielded hesperidin (0.547 mM) as the acceptor of the CGTase reaction and the corresponding amount of glucose (0.529 mM). The molar ratio of hesperidin to glucose was 1:0.97. Hydrolysis by α-glucosidase gave the same results, which molar ratio was 1:1.13. But β-glucosidase could not hydrolyze glycoside C. The results suggested that glycoside C was hesperidin-α-D-mouoglucopyranoside.

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.

Table. Ratios of Partially Methylated Alditol Acetates from Glycoside C.

Alditol acetate	Glycoside C	Hesperidin	Maltosyl-β-cyclodextrin
2,3,4,6-tetra-*O*-methyl-glucitol acetate	1.00*1)	-	1.00
2,3-di-*O*-methyl-glucitol acetate	1.07	-	1.14
2,3,4-tri-*O*-methyl-glucitol acetate	-	0.77	-
2,3,6-tri-*O*-methyl-glucitol acetate	-	-	7.29
2,3,4-tri-*O*-methyl-rhamnitol acetate	D*2)	D	-

*1); Numeral values represent the molar ratios of partially methylated alditol acetates from each sample, when the amount (mol) 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.[39]

*2); The peak was detected and the molar ratio was not calculated.

From FAB-MS, enzymatic and methylation analyses, glycoside C was identified as 4^G-α-D-glucopyranosyl hesperidin (Fig. 5).

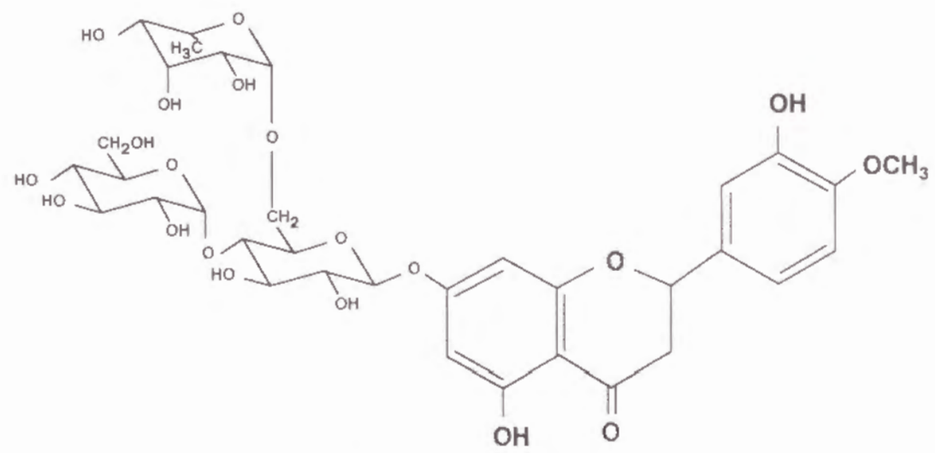


Fig. 5. Structure of glycoside C.

Structures of glycosides A and B

To identify the strncture 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 (hesperidin). In addition, glycoside B was hydrolyzed with glucoamylase or α-glucosidase in the same manner as described in Enzymatic analysis section of glycoside C. 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.

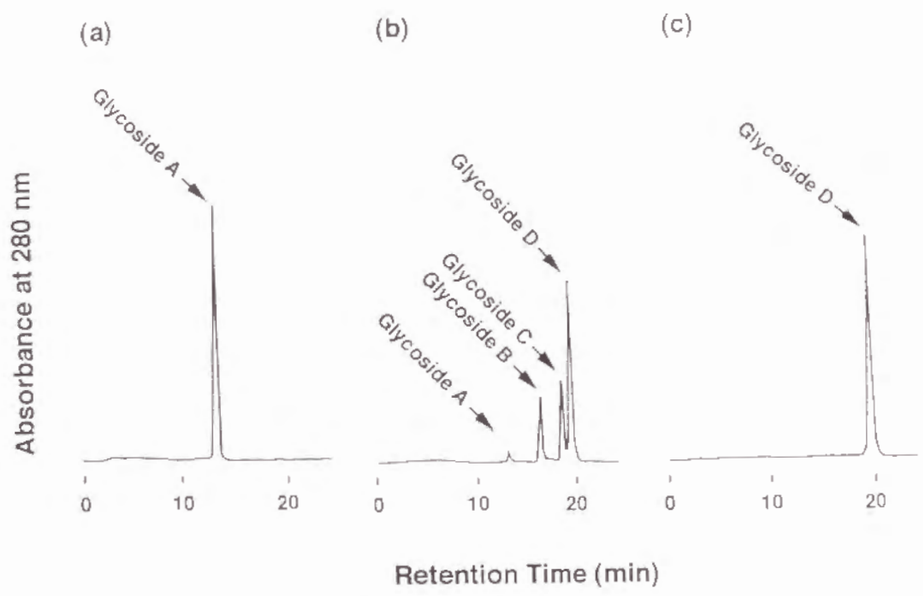


Fig. 6. Glucoamylase Treatment of Glycoside A.

Purified glycoside A was treated with glucoamylase and the products were analyzed by HPLC on an ODS at (a), 0; (b), 2; and (c), 16 h.

Apparent solubility of glycosides B and C

Purified glycosides B (47 mg) and C (23 mg) was dissolved in 400 and 200 μl of H_2O. Their apparent solubilities were measured by HPLC on an ODS described above. Glycosides B and C could be dissolved in H_2O 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 H_2O.

Stabilization of the pigmentation of 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 25% 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.

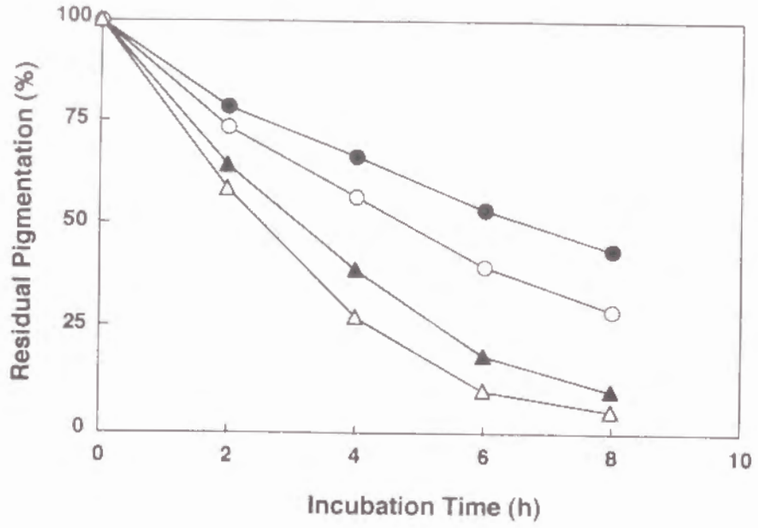


Fig. 7. Effects of Hesperidin Glycosides on Stabilizing the Pigmentation of Crocin.

Solutions containing 0.01%; (▲), 0.05%; (○), and 0.1%; (●) hesperidin glycoside mixture were tested under the conditions described in Materials and Methods. Control solution (△) did not contain hesperidin glycoside mixture.

Discussion

There are many reports on transglycosylation by CGTase,[5,23-29] but no reports on transglycosylation at alkaline pHs. The author have described transglycosylation to various flavonoids at alkaline pHs (Chapter I, section 1), because they were more soluble at alkaline pHs than at neutral or acidic pHs. In this section, the author tried transglycosylation 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 were 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 glucosides 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-l, 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.[28] 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 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 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).

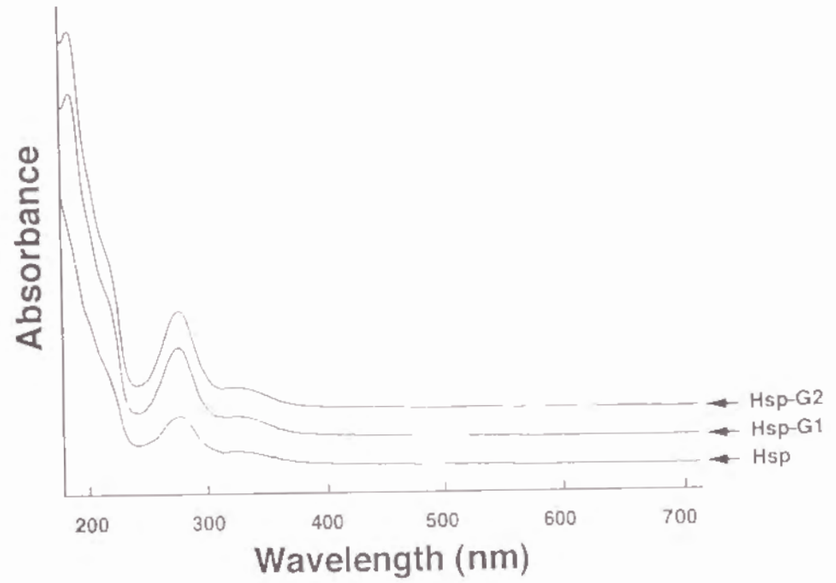


Fig. 8. Comparison of Absorption Spectra of Hesperidin Mono, Diglucoside and Hesperidin.

Hesperidin mono (Hsp-G1; 0.025 mM) and diglucoside (Hsp-G2; 0.025 mM) and hesperidin (Hsp; 0.018 mM) were dissolved in H_2O and assayed.

In addition, they do not have strong spectra in visible light (Fig. 8). Furthermore, they have no 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 hypotensive effects against blood pressure, so solubilized so-called vitamin P may be expected. Precise studies in this field are now needed.

Summary

Cyclodextrin glucanotransferase from an alkalophilic *Bacillus* species produced hesperidin monoglucoside and a series of its oligoglucosides by the transglycosylation reaction with hesperidin as an acceptor and soluble starch as a donor. The formation of the glycosides was more effective at alkaline pHs than at neutral or acidic pHs, because of higher solubility of the acceptor.

Structure of the purified monoglucoside was identified as 4^{G}-α-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 found to have stabilizing effect on the yellow pigment crocin, from fruits of *Gardenia jasminoides*, against ultraviolet radiation.

Section 3. Improvement for Synthesis of Hesperidin Glycosides by Cyclodextrin Glucanotransferase using Cyclodextrins and α-Rhamnosidase, and Stabilization of the Pigmentation of Various Natural Pigments

Introduction

In previous section, the author described transglycosylation of flavonoids by CGTase from an alkalophilic *Bacillus* species in alkaline pH range (Chapter I, section 1). In addition, the author also described the optimum condition for the synthesis of hesperidin glycosides, the characterization of the glycosides, and utilization of them for stabilizing natural pigment (crocin, yellow pigment from fruits of *Gardenia jasminoides*) against ultraviolet radiation (Chapter I, section 2). In order to apply hesperidin glycosides for an industrial use, as a food additive which stabilizes the pigmentation of food, it is necessary to obtain the simple and effective method for synthesis of them.

In this section, the author improved the method for the synthesis of them using cyclodextrins (CDs) and α-rhamnosidase from *Aspergillus niger*, and described their stabilizing effects on various natural pigments against ultraviolet radiation.

Materials and Methods

Chemicals. Hesperidin, hesperetin-7-glucoside, and hesperetin were purchased from Sigma Chemical Co., Ltd, and soluble starch was done from E. Merck. α-CD, β-CD and γ-CD were purchased from Wako Pure Chemical Industries, Ltd. K-100 (mixture of α-CD and β-CD; 2:1, purity of CDs; 98%) was gifted from Ensuiko Sugar Refining Co., Ltd. Natural pigments such as annatto (from seeds of *Bixa orellana* L.), betanine (from roots of *Beta vulgaris* L.), phycocyanin (from *Spirulina* sp.), carminic acid (from *Dactylspiums coccus* Costa), laccaic acid (from *Laccifer lacca* Kerr.), riboflavin and chlorophyll were donated from Glico Foods 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 (Chapter I, section 1). β-Amylase from sweet potato was purchased 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. Hesperidinase (commercial enzyme of α-rhamnosidase from *Aspergillus niger*) or naringinase (commercial enzyme mixture of α-rhamnosidase and β-glucosidase from *A. niger*) were gifted from Tanabe Seiyaku Co., Ltd. α-Rhamnosidase activity was assayed according to the method of Horiuchi et al.[40] with a slight modification of a HPLC condition. The activity was assayed using hesperidin or naringin as a substrate by measuring the decrease in the amount of substrate with 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. One unit of the enzyme was defined as the amount of enzyme that hydrolyzed 1 mg hesperidin or naringin at 40 °C and pH 4 for 1 h. β-Glucosidase activity was assayed using *p*-nitrophenyl β-D-glucoside as a substrate by measuring the increase in the amount of *p*-nitrophenol, according to the method of Grover et al,[41] and one unit of the enzyme was defined as the amount of enzyme that produced 1 mmole *p*-nitrophenol at 37 °C and pH 5 for 1 min. On this condition, one unit of naringinase contained 0.005 units of β-glucosidase.

Transglycosylation. The reaction mixture (1 ml) containing 0.5% (8.2 mM) hesperidin as an acceptor, 0-5% soluble starch or each CD as a donor and 2 units of CGTase was incubated at 40 °C for 16 h at pH 5 and 10. The reaction was stopped by boiling for 5 min, and centrifuged at 5,000 *g* for 5 min to remove insoluble hesperidin. Transfer products and unreacted hesperidin in the supernatant were determined by HPLC on an ODS described above. The amount of hesperidin in the reaction mixture without enzyme was also determined by HPLC on an ODS as soluble one. The amount of hesperidin glycosides was calculated as subtraction of the amount of residual hesperidin from that of soluble one.

Preparation of hesperidin glycosides. The reaction mixture (20 l) containing 0.25% (4.1 mM) hesperidin, 5% soluble starch, and 40,000 units of

CGTase was incubated at 40 °C and pH 10 for 16 h. After heating at 90 °C for 10 min, the reaction mixture was applied onto 1st Amberlite XAD-16 column (Organo Co., Ltd.). The resin adsorbed hesperidin glycosides was washed with H_2O, and hesperidin glycosides and unreacted hesperidin were eluted with 50% ethanol. After concentrated *in vacuo*, the eluate was incubated with 300 units of β-amylase at 40 °C and pH 5.5 for 16 h. The reaction mixture was boiled for 5 min, and concentrated *in vacuo*. The concentrate was treated with 8,000 units of naringinase, and applied onto 2nd Amberlite XAD-16 column. The resin adsorbed hesperidin glycosides was washed with H_2O, and hesperidin glycosides were eluted with 50% ethanol. After concentrated *in vacuo*, the concentrate was applied onto Sephadex LH-20 column eluted with 50% ethanol at a flow rate of 2 ml/min at room temperature. The fractions containing hesperidin glycosides were collected, and they were obtained as a powder by lyophilization.

Stabilization of the pigmentation of various pigments. The solution containing 0.05-0.1% natural pigment and 0.01-0.1% hesperidin glycoside mixture (a mixture of hesperidin mono and diglucoside, 1:1, w/w) was stood at 4 °C under 12,000 lux. The solution without hesperidin glycoside mixture was used as a control. The absorbance of each solution was measured at various intervals. The residual pigmentation was expressed as the percentage of each absorbance measured, which was based on that of starting solution.

Results

Effects of glycosyl donors and pHs on the synthesis hesperidin glycosides

To investigate effects of glycosyl donors and pHs on transglycosylation, the reaction mixture (1 ml) containing 0.5% (8.2 mM) hesperidin as an acceptor, 5% soluble starch or 5% each CD (α-, β-, γ-CD, and K-100) as a donor, and 2 units of CGTase from an alkalophilic *Bacillus* sp. was incubated at 40 °C for 16 h at pH 5 and 10.

At pH 5, as shown in Fig. 1, the amount of soluble hesperidin using β-CD as a donor was about 4 times greater than that using soluble starch, which seemed to be due to make an inclusion complex. In transglycosylation, according to increase the amount of soluble hesperidin, that of hesperidin glycosides formed by CGTase was increased.

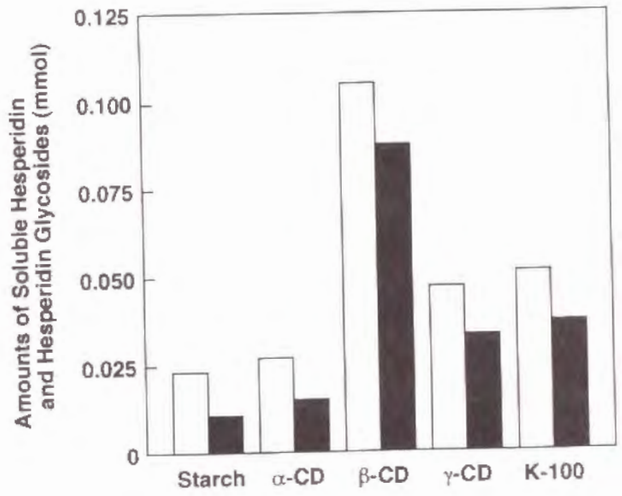


Fig. 1. Effects of Glycosyl Donors on the Synthesis of Hesperidin Glycosides at pH 5.

(□), the amount of soluble hesperidin; (■), the amount of hesperidin glycosides formed.

At pH 10, as shown in Fig. 2, transglycosylation reaction showed the same tendency as that at pH 5, and CDs found to be able to make an inclusion complex even at alkaline pH. The amounts of soluble hesperidin and hesperidin glycosides using β-CD as a donor were increased about 4-5 times greater than those using soluble starch. As a result, β-CD was found to be the most effective donor of the five, and next was K-100, γ-CD, α-CD, soluble starch, in turn.

Comparing the results at pH 5 and 10, the amounts of soluble hesperidin and hesperidin glycosides using β-CD at pH 10 was about 30 times greater than those using soluble starch at pH 5.

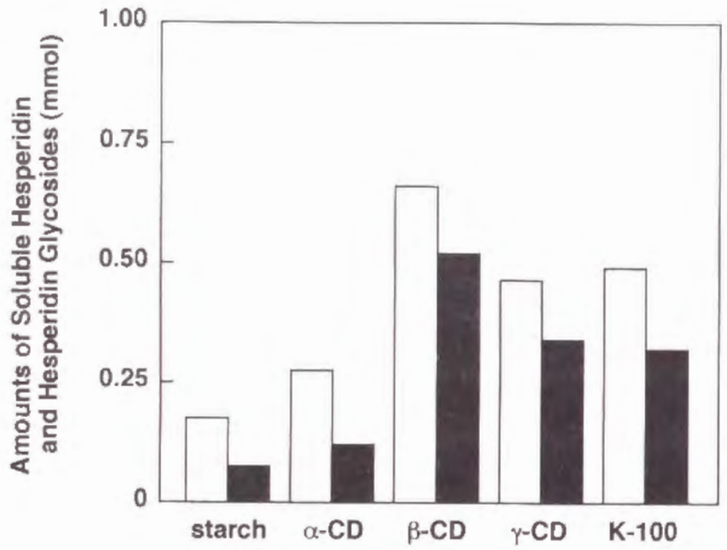


Fig. 2. Effects of Glycosyl Donors on the Synthesis of Hesperidin Glycosides at pH 10.

(□), the amount of soluble hesperidin; (■), the amount of hesperidin glycosides formed.

In order to investigate the effects of combination with soluble starch and β-CD on the synthesis of hesperidin glycosides, the reaction mixture (1 ml) containing 0.5% hesperidin, 0-5% soluble starch with 2% β-CD, and 2 units of CGTase was incubated at 40 °C for 16 h at pH 10. As shown in Fig. 3, both the amounts of soluble hesperidin and hesperidin glycosides formed were not almost affected by the concentration of soluble starch.

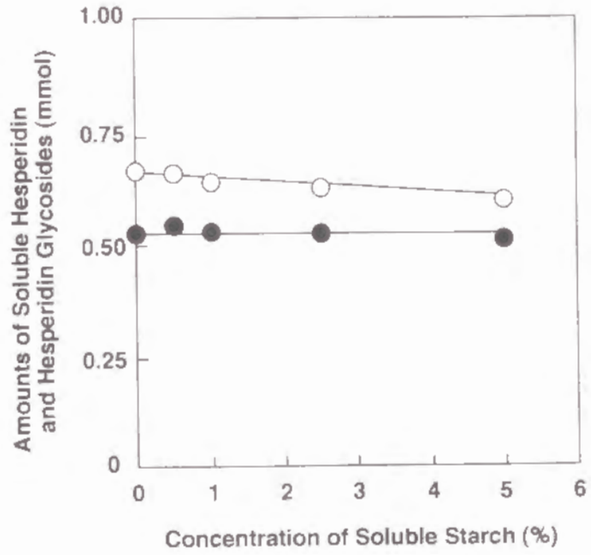


Fig. 3. Effects of Combination with Soluble Starch and β-CD on the Synthesis of Hesperidin Glycosides.

(○), the amount of soluble hesperidin; (●), the amount of hesperidin glycosides formed.

Effects of concentration of β-CD on the synthesis of hesperidin glycosides

As β-CD was found to be the best glycosyl donor, the author investigated the effects of concentration of that on the synthesis of hesperidin glycosides. The reaction mixture (1 ml) containing 0.5% hesperidin, 0-5% β-

CD, and 2 units of CGTase was incubated at 40 °C and pH 10 for 16 h. As shown in Fig. 4, when the concentration of β-CD was increased up to 2%, the amount of hesperidin glycosides was increased proportionally to that of soluble hesperidin. At more than 2% β-CD, they were not increased any more. In this condition, 2% β-CD seems to be enough for the synthesis of hesperidin glycosides.

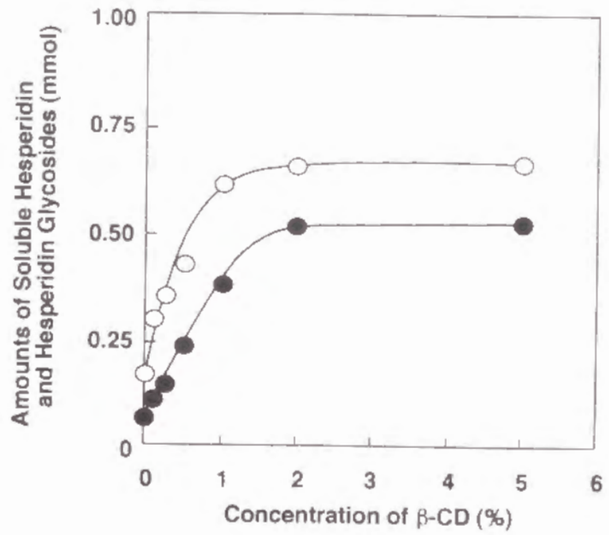


Fig. 4. Effects of Concentration of β-CD on the Synthesis of Hesperidin Glycosides.

(○), the amount of soluble hesperidin; (●), the amount of hesperidin glycosides formed.

Preparation of hesperidin glycosides with α-rhamnosidase treatment

In preparation steps of hesperidin glycosides, there were two problems to be solved. First was that CGTase produced many kinds of transfer products such as hesperidin monoglucoside and oligoglucosides. Using β-amylase, the

author tried to hydrolyzed hesperidin oligoglucosides into hesperidin tri and diglucosides. Second was the difficulty to separate unreacted hesperidin from hesperidin monoglucoside, because the properties of them were seemed to be relatively similar. Using α-rhamnosidase such as hesperidinase and naringinase, the author tried to convert unreacted hesperidin to hesperetin-7-glucoside or hesperetin, more hydrophobic and lower molecular weight compounds.

The reaction mixture containing 0.25% hesperidin, 5% soluble starch, and 40,000 units of CGTase was incubated at 40 °C and pH 10 for 16 h. After heating at 90 °C for 10 min, the mixture was applied onto 1st Amberlite XAD-16, and the eluate from this resin was treated with β-amylase. And the resultant reaction mixture was treated with hesperidinase or naringinase. Fig. 5 showed the effects of naringinase treatment. The mixture was incubated with 40 units/ml naringinase at 40 °C and pH 4 for 0.5-7 h. The amount of hesperidin was decreased rapidly to convert into hesperetin-7-glucoside and hesperetin. During this treatment, the amount of hesperidin monoglucoside increased and that of hesperidin diglucoside decreased. As mono and diglucoside were not hydrolyzed with hesperidinase (data not shown), this seemed to be due to some amylases or glycosidases contaminated in naringinase. However, since naringinase contained relatively lower β-glucosidase activity, it could not hydrolyze hesperetin-7-glucoside into hesperetin completely.

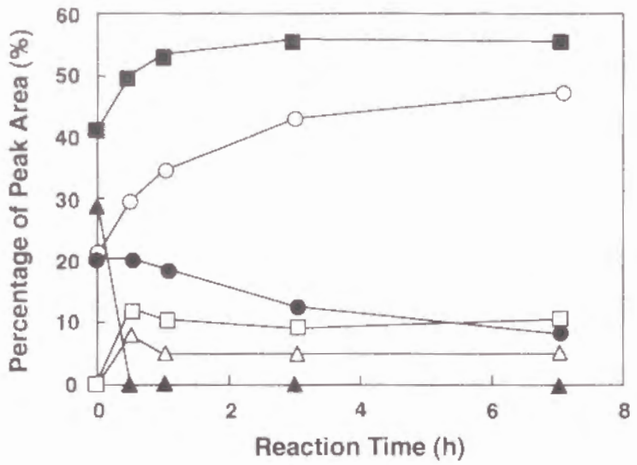


Fig. 5. Naringinase Treatment of Hesperidin Glycosides after Treated by β-Amylase.

(○), Hsp-G1 (hesperidin monoglucoside); (●), Hsp-G2 (hesperidin diglucoside); (■), Hsp-G1+Hsp-G2; (▲),Hsp (hesperidin); (△), Hspt-7-G (hesperetin-7-glucoside); (□), Hspt (hesperetin).

As resultant hesperetin has more hydrophobic property than hesperetin-7-glucoside, it was absorbed on 2nd Amberlite XAD-16 tightly. Then, the fraction eluted with 50% ethanol from this resin was contained only hesperidin glycosides and hesperetin-7-glucoside, and they were concentrated and applied onto Sephadex LH-20. As shown in Fig. 6, hesperetin-7-glucoside originated from unreacted hesperidin was easily separated from hesperidin mono and diglucoside by this step, because of its lower molecular weight and more hydrophobic property.

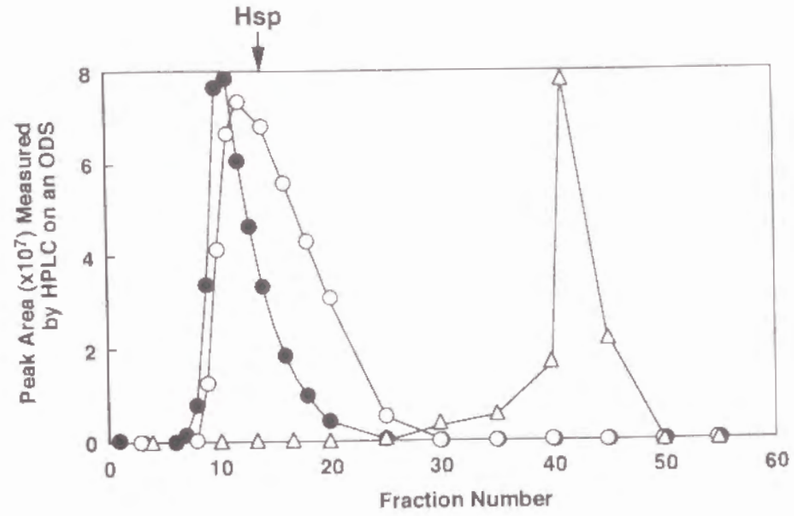


Fig. 6. Profile of Sephadex LH-20 Chromatography.

(○), Hsp-G1; (●), Hsp-G2; (△), Hspt-7-G; (←Hsp), the eluting point of hesperidin, when it was applied onto this column.

The purity of the fraction gained Sephadex LH-20 step was analyzed by HPLC on an ODS described above, and TLC which was done with Silica gel 60 (E. Merck) and solvent system; chloroform-methanol-H_2O (65:35:10, v/v/v, lower layer) detecting absorbance at 253 nm and spraying 50% (v/v) H_2SO_4 followed by heating at 130 °C for 5 min. Authentic samples of hesperidin mono and diglucoside were purified as described in Chapter I, section 2. The mixture consisted of hesperidin monoglucoside and diglucoside. Its molar ratio was estimated about 1:1 by HPLC on an ODS (Fig. 7), and TLC analysis was confirmed this result. As a result, 14.8 g of hesperidin glycoside mixture was obtained from 50 g of hesperidin.

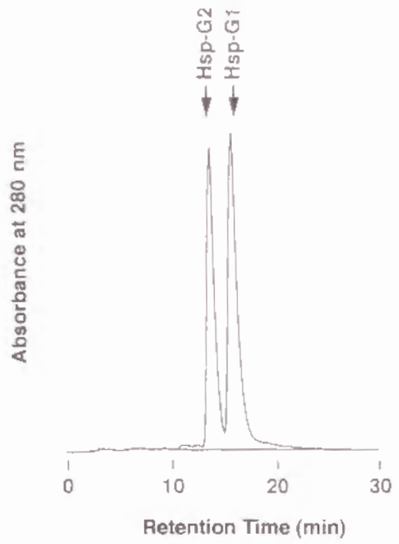
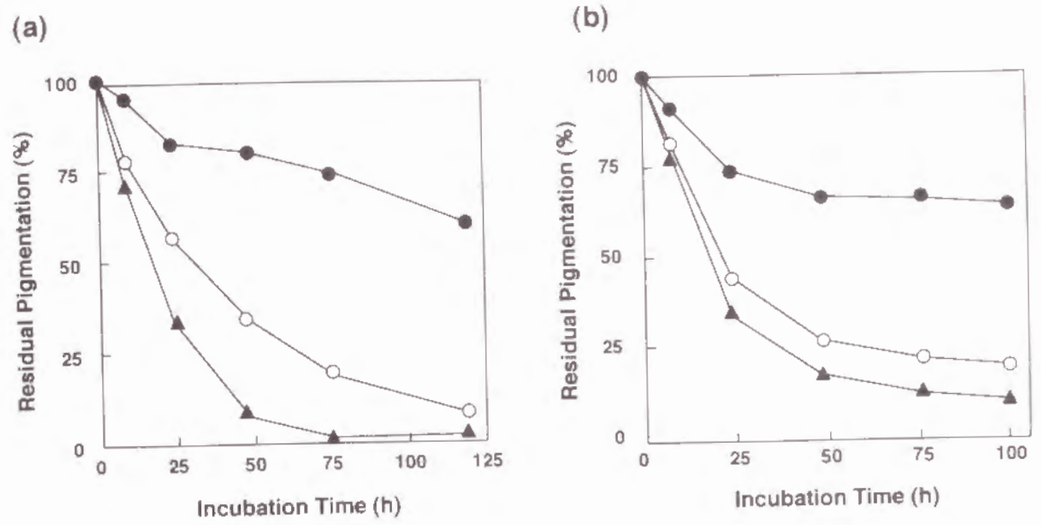


Fig. 7. HPLC Profile of Prepared Hesperidin Glycoside mixture.

Stabilization of the pigmentation of various pigments by hesperidin glycosides

Stabilizing effects of hesperidin glycoside mixture on the pigmentation of various natural pigments against ultraviolet light was investigated. Seven pigments such as annatto, carminic acid, laccaic acid, betanine, phycocyanin, riboflavin and chlorophyll were used. Typical result was in the case of annatto pigment (Fig. 8-a). The residual pigmentation of control solution was decreased rapidly and it was about one-third times lower than that of starting one by 24 h. It reached almost zero at 72 h. On the other hand, the residual pigmentation of the solutions containing 0.01 and 0.1% hesperidin glycoside mixture was more stable than that of control one. The residual pigmentation of the solution containing 0.1% hesperidin glycosides was retained more than 70% of that of starting one even at 120 h. As shown in Fig. 8-b~f,

stabilization of the pigmentation of other pigments was the same tendency as that of annatto pigment.



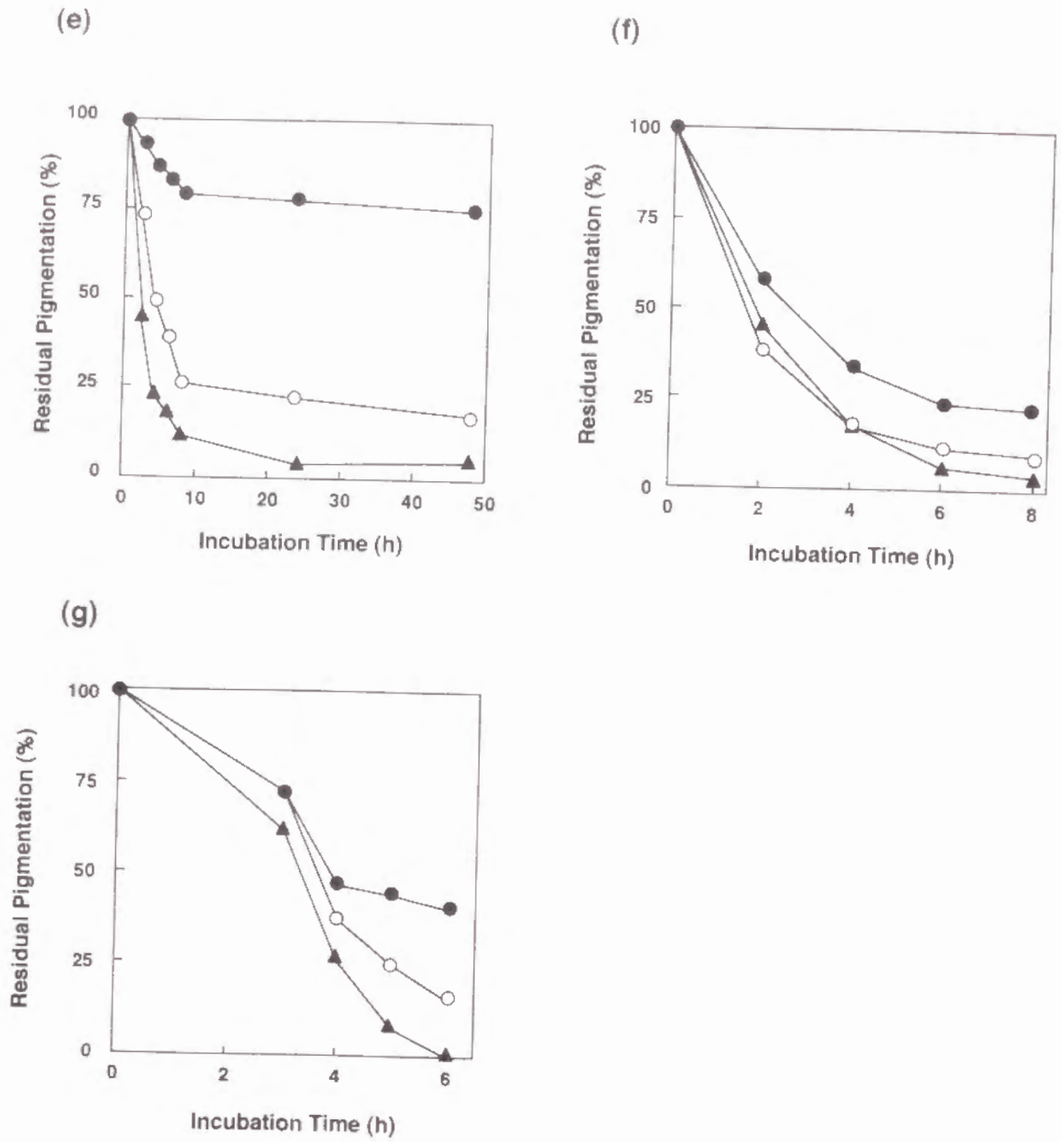


Fig. 8. Stabilization of the Pigmentation of Various Pigments by Hesperidin Glycosides.

(a), annatto (0.025% in H_2O); (b), laccaic acid (0.188% in sodium carbonate buffer (pH 9)); (c), phycocyanin (0.1% in H_2O); (d), betanine (0.25% in citrate buffer (pH 4)); (e), carminic acid (0.375% in sodium carbonate buffer (pH 9)); (f), riboflavin (0.25% in H_2O); (g), chlorophyll (0.025% in H_2O).

(▲); 0%, (○); 0.01% and (●); 0.1% hesperidin glycoside mixture was added to each pigment solution, and stood at 4 °C under 12,000 lux.

Discussion

Recently, the demand of natural pigments is increasing, because of increasing consciousness for safety in food materials. However, there are some problems to be solved, and one of them is low stability of the pigmentation. As diminishing the pigmentation of food was extremely reduced the value as a merchandise, the natural pigments are expected to improve their stability for an industrial use. Therefore, it is important to retain the pigmentation in food manufactures. For this purpose, the author produced hesperidin glycosides by transglycosylation with CGTase and tested them for a practical use described in Chapter I, section 2. When pigment solution containing hesperidin glycosides were revealed to ultraviolet light, hesperidin glycosides found to stabilize the pigmentation. It might be caused that the glycosides absorbed ultraviolet light, and reduced the influences of ultraviolet light on pigments. In addition, the glycosides had almost the same ultraviolet absorption spectra as hesperidin, and do not have absorption spectra in visible light range. Furthermore, they have almost no specific flavor and taste. These results showed the possibility of hesperidin glycosides as a stabilizer of natural pigments in food against ultraviolet light.

In order to produce hesperidin glycosides simply and effectively, the author tried to synthesize hesperidin glycosides using CDs and α-rhamnosidase. As known well, high concentration of acceptor in a reaction mixture is desired in transglycosylation. In this section, to obtain much more amount of hesperidin glycosides, solubilization of hesperidin using CDs was attempted. The solubilization and transglycosylation of hesperidin using CDs at

pH 10 were much greater than that without CDs at pH 5 (Fig. 1 and 2). Comparing α-, β-, and γ-CD for solubilization and transglycosylation of hesperidin, β-CD was the best donor. It was reported that β-CD could form a good inclusion complex with hesperidin, and ^{1}H-NMR analysis showed that β-CD formed an inclusion complex, not with the rhamnose moiety, but with phenyl ring (B-ring) of hesperidin.[42] In addition, comparing of the effects of α-, β-, and γ-CD on solubilization of hesperidin, β-CD was found to be the most effective of the three.[43] These previous studies supports the results on transglycosylation to hesperidin using β-CD.

From these results, β-CD was thought to work both as a glycosyl donor and as a solubilizer of acceptor by an inclusion effect.

In order to obtain hesperidin glycosides not containing unreacted hesperidin, the author tried to treat the glycosides with α-rhamnosidase. In naringinase (mixture of α-rhamnosidase and β-glucosidase) treatment, α-rhamnosidase was effectively hydrolyzed hesperidin to yield hesperetin-7-glucoside, and coexisted β-glucosidase also hydrolyzed hesperetin-7-glucoside to do hesperetin. But hesperidin glycosides were not affected with these two enzymes. It seems that these enzymes could not attack hesperidin glycosides because of the structural inhibition of their glucose moieties transferred by CGTase. It is the advantage of this treatment that α-rhamnosidase hydrolyzed only unreacted hesperidin without any effects to hesperidin glycosides formed by CGTase. As hesperidin glycosides were more hydrophilic and had higher molecular weight, and both hesperetin-7-glucoside and hesperetin were more hydrophobic and lower molecular weight than unreacted hesperidin,

hesperidin glycosides were easily separated from unreacted hesperidin. Naringinase treatment found to be effective for preparation of hesperidin glycosides.

As for the stabilization of pigments, there were the differences of the stabilizing property against ultraviolet light between each pigment tested. Annatto, carminic acid, laccaic acid, phycocyanin and betanine were relatively stable. On the other hand, crocin (Chapter I, section 2), riboflavin and chlorophyll were relatively unstable, and their pigmentation were decreased rapidly in several hours. The results shown here revealed that hesperidin glycosides were effective on the stabilization of various natural pigments, and suggested that the glycosides might be applied for food ingredient.

In addition, the simple method for purification of hesperidin from mandarin orange peel residue, which is the waste in juice industries, has been developed by Miyake et al,[36,37] and it could easily and cheaply gain in a large quantity. Hesperidin glycosides may be easily and cheaply obtained with both the method of Miyake et al. and the improved method in this study, and utilized as food additives such as stabilizer of pigments.

Summary

Improved method for synthesis of hesperidin glycosides was developed with both β-cyclodextrin (β-CD) and α-rhamnosidase treatments. The reaction, which 0.5% (8.2 mM) hesperidin as an acceptor, 5% soluble starch as a donor, and 2 units/ml of cyclodextrin glucanotransferase (CGTase) from an alkalophilic *Bacillus* species was incubated at 40 °C and pH 10 for 16 h, was used as a standard reaction.

In transglycosylation reaction, both β-CD and alkaline pH were much effective for solubilizing hesperidin, and the amount of hesperidin glycosides formed by CGTase with 5% β-CD as a donor at pH 10 was about 30 times greater than the amount of them with soluble starch at pH 5. In purification steps, α-rhamnosidase hydrolyzed unreacted hesperidin into lower molecular weight and more hydrophobic compounds (hesperetin-7-glucoside), and it did not hydrolyzed hesperidin glycosides. Therefore, hesperidin glycosides were easily separated from unreacted hesperidin by following Sephadex LH-20.

Various kinds of natural pigments were stabilized against ultraviolet radiation by prepared hesperidin glycosides.

Section 4. A New Method for Precipitation of Various Glycosides with Cyclodextrin Glucanotransferase from *Bacillus macerans*

Introduction

Cyclodextrin glucanotransferase (CGTase) catalyzes not only intramolecular (cyclization, the conversion of starch to cyclodextrin (CD)) and intermolecular (coupling and disproportionation, the transfer glycosyl residues to the suitable acceptor) transglycosylation, but also hydrolysis of starch and CD.[44] Since Tilden and Hudson discovered this enzyme in *Bacillus macerans*,[45] CGTase has been found in many microorganisms,[12] i.e., *B. circulans*, *B. megaterium*, *B. stearothermophilus*, *B. ohbensis*, *B. subtilis*, alkalophilic *Bacillus* sp., *Klebsiella pneumoniae*, and *B. coagulans*.[46] There have been many studies on transglycosylation by CGTase, i.e., transglucosylation to glucose,[23,24] sucrose,[25] stevioside,[5,26] rubusoside,[27] rutin,[28] and L-ascorbic acid,[29] etc.

These studies dealt with transglycosylated products that were water-soluble, that is, they were mono or oligoglucosylated ones. However, there has been only one report on the synthesis of polyglucosylated products, which was amylose production from α-CD by CGTase.[47]

In this section, the author described transglycosylation to various glycosides including salicin, rubusoside, stevioside, geniposide, and rutin glucoside by CGTase, which products had higher molecular weight than ever

reported and precipitated at 4 °C spontaneously. The author also showed the possibility of selective precipitation of various glycosides from crude extracts.

Materials and Methods

Chemicals. Salicin (2-[hydroxymethyl]phenyl-β-D-glucoside) was purchased from Sigma Chemical Co. Rubusoside (13-*O*-β-D-glucosyl-19-*O*-D-glucosyl-steviol) was a gift from Dr. Kitahata (Osaka Municipal Technical Research Institute). Stevioside (13-*O*-β-sophorosyl-19-*O*-β-D-glucosyl-steviol) was purchased from Wako Pure Chemical Industries, Ltd. Geniposide (genipin-β-D-glucoside) and rutin glucoside (4^G-α-D-glucopyranosyl rutin) were obtained from Glico Foods Co., Ltd. and San-ei Chemical Industries, Ltd., respectively.

Enzymes. CGTases from *B. macerans* and *B. stearothermophilus* were obtained from Amano Pharmaceutical Co., Ltd. and Hayashibara Biochemical Co., Ltd., respectively. CGTase from *B. circulans* was donated from Dr. Kitahata, and CGTase from *B. megaterium* was purified to a homogeneous state as described previously.[48] CGTase activity was assayed using soluble starch (E. Merck) as a substrate by measuring the decrease in iodine-staining power, as described in Chapter I, section 1. Glucoamylase from *Rhizopus* sp. was purchased from Toyobo Co., Ltd.

Transglycosylation and the yield of polyglucosylated products. Four CGTases (8 units/ml) from *B. macerans*, *B. stearothermophilus*, *B. circulans*, and *B. megaterium* were separately incubated with 2.9 mM salicin as an acceptor and 70 mM α-CD as a donor at 40 °C and pH 5.5 for 4 h. After the reaction mixture was boiled at 100 °C for 5 min to inactivate the enzyme,

the mixture was left at 4 °C for 6 h. The resultant precipitate was washed 3 times with cold distilled water and dissolved in 0.5 N NaOH. After the solution was neutralized, 10 units/ml of glucoamylase was added and incubated at 40 °C for 16 h. The acceptor (salicin) in the hydrolysate was measured by HPLC on an ODS column (E. Merck) eluted with methanol-H_2O (25:75, v/v) at a flow rate of 0.5 ml/min at 60 °C, detecting absorbance at 270 nm. The yield of acceptor in the precipitate or supernatant was expressed as the percentage of acceptor detected in each fraction based on the amount of the acceptor applied.

Assay for transglycosylated products. The yield of each glycoside was measured by HPLC and a photometrical assay; for salicin, described above; for rubusoside and stevioside, ODS column (E. Merck) eluted with methanol-H_2O (70:30, v/v) at a flow rate of 0.5 ml/min at 60 °C, detecting absorbance at 213 nm; for geniposide, ODS column eluted with methanol-H_2O (25:75, v/v) at a flow rate of 0.5 ml/min at 60 °C, detecting absorbance at 240 nm; for rutin glucoside, the amount of rutin glucoside was measured by the absorbance at 420 nm as rutin by the method described previously.[28]

Results

Comparison of the yield of polyglucosylated products

Four CGTases from *B. macerans*, B. *stearothermophilus*, *B. circulans*, and *B. megaterium* were tested for their ability to transglycosylate salicin. At the initial stage of each reaction, a series of transglycosylated products were detected by HPLC analysis. After left at 4 °C for 6 h, polyglucosylated products of salicin were precipitated, on the other hand, mono and oligoglucosylated ones were in the supernatant. The yield of polyglucosylated glycosides by enzyme from *B. macerans* was the highest. It was about 50% and those of the other enzymes from *B. stearothermophilus*, *B. circulans*, and *B. megaterium* were about 2%, 15 %, and 15 %, respectively. Residual salicin or α-CD in each reaction mixture was detected at less than 10% of total salicin or α-CD applied. In this study, because of its higher yield of polyglucosylated glycosides, the author used the CGTase from *B. macerans*.

Optimum conditions of transglycosylation reaction

1. Effects of enzyme concentration.

The effects of enzyme concentration on synthesis of polyglucosylated glycosides were examined. Each enzyme solution (2-128 units/ml) was added to the mixture of 2.9 mM salicin and 70 mM α-CD, and incubated at 40 °C and pH 5.5 for 4 h. As shown in Fig. 1, the polyglucosylated glycosides were observed to form at low enzyme concentrations, and at higher concentrations of enzyme they disappeared gradually. At 8 units/ml, the synthesis of the polyglucosylated glycosides was the highest and the yield was 44.6%.

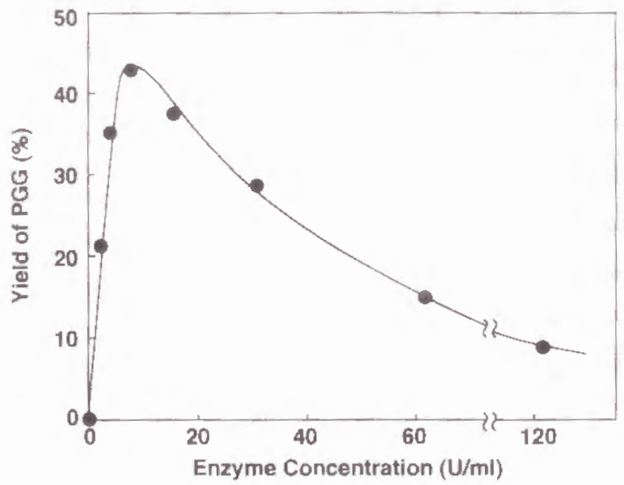


Fig. 1. Effects of Enzyme Concentration on Synthesis of Polyglucosylated Glycosides (PGG).

2. Effects of reaction time.

The effects of reaction time were also examined. Eight units/ml of enzyme was added to the reaction mixture described above and incubated at 40 °C and pH 5.5 for several hours. As shown in Fig. 2, the synthesis of polyglucosylated glycosides was the highest at 4 h of incubation, and gradually decreased with incubation time.

3. Effects of temperature.

To examine the effects of temperature, the standard reaction was done at various temperatures for 4 h. As shown in Fig. 3, the synthesis of polyglucosylated glycosides was the highest of all at 30 °C and the yield was 57.7%.

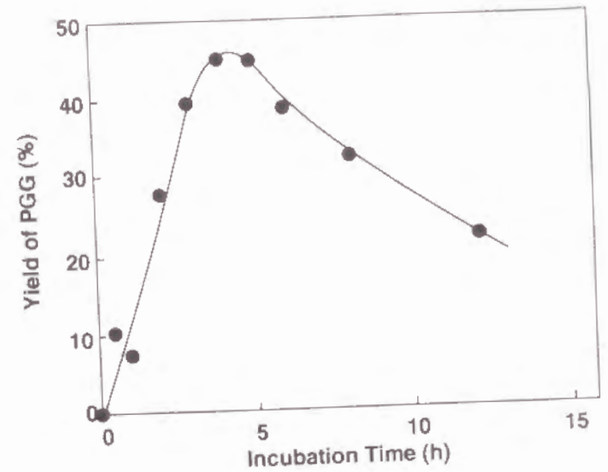


Fig. 2. Effects of Incubation Time on Synthesis of Polyglucosylated Glycosides.

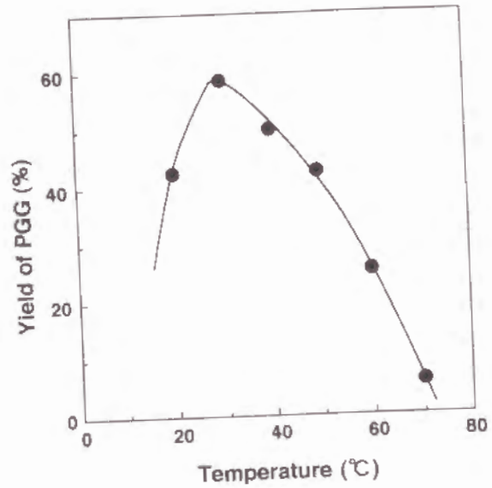


Fig. 3. Effects of Temperature on Synthesis of Polyglucosylated Glycosides.

4. Effects of acceptor/donor ratio.

The effects of the salicin (acceptor)/α-CD (donor) (molar ratio) on the synthesis of polyglucosylated glycosides were studied. Reaction mixtures containing 70 mM α-CD, 8 units/ml CGTase, and various concentrations of

salicin were incubated at 40 °C and pH 5.5 for 4 h. When these reaction mixtures were left at 4 °C for 6 h, a series of transglycosylated salicins were detected in both precipitate and supernatant. As the salicin/α-CD ratio decreased, as shown in Fig. 4, transglycosylated products in the precipitate (polyglucosylated glycosides) increased; in contrast, soluble glycosides (mono and oligoglucosylated ones) decreased. Residual salicin was less than 10% of that applied. When 100 ml of reaction mixture including 2.9 mM salicin (83 mg), 70 mM α-CD, and 8 units/ml CGTase were incubated at 40 °C for 4 h, after the precipitate was treated with glucoamylase, the yield of salicin was 37 mg. This results might be applied to a new method for preparing glycosides from crude extracts through precipitate formation.

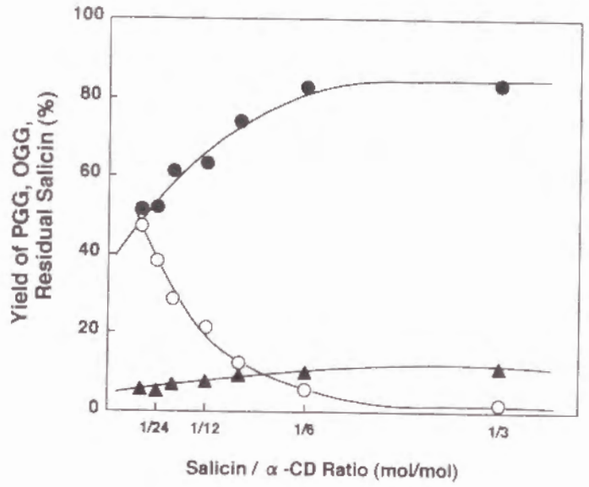


Fig. 4. Effects of Salicin/α-CD Ratio on Synthesis of Polyglucosylated Glycosides.

(○), the yield of polyglucosylated glycosides (PGG); (●), the yield of oligoglucosylated glycosides (OGG); (▲), the residual amount of salicin.

Acceptor specificity

To use this precipitation method for various glycosides, the synthesis of polyglucosylated glycosides was examined. The yield of each glycoside was measured by HPLC and a photometrical assay described above. The results are summarized in Table. All glycosides used were effective acceptors and resultant polyglucosylated glycosides were precipitated, when they were left at 4 °C for 6 h. After each precipitate was treated with glucoamylase as described above, the yield of each glycoside was approximately 30-50% based on the amount applied.

Table. Yield of Various Glycosides through Polyglucosylated Glycosides

Glycosides	Yield (%)
Salicin	51.8
Rubusoside	32.7
Stevioside	33.5
Geniposide	43.8
Rutin glucoside	51.1

The mixture of each glycoside (2.9 mM), α-CD (70 mM), and CGTase (8 units/ml) from *B. macerans* was incubated at 40 °C and pH 5.5 for 4 h and left at 4 °C for 6 h. After the resultant precipitate was treated with glucoamylase, yield of each glycoside was measured as described in Materials and Methods.

Discussion

CGTase from *B. macerans* catalyzed transglycosylation reaction of α-CD and glycosides such as salicin successively to synthesize the polyglucosylated products, which precipitated at 4 °C spontaneously. Although other 3 enzymes also catalyzed transglycosylation reaction similarly as enzyme from *B. macerans* did, the amount of the polyglucosylated ones was smaller than that synthesized by enzyme from *B. macerans*.

The optimum condition of the synthesis of polyglucosylated products was that 8 units/ml of CGTase from *B. macerans* was incubated with 2.9 mM salicin and 70 mM α-CD at 30 °C and pH 5.5 for 4 h. The yield of polyglucosylated products was approximately 60% based on the amount of salicin used.

Substrate specificity on the CGTase reaction was also examined. All tested glycosides became good substrates for CGTase from *B. macerans*. Therefore, various glycosides could be converted to polyglucosylated products.

Many glycosides were found as biologically active substances in plant extracts like Chinese medicines, but purification is very difficult on an industrial scale. Making use of this phenomenon found in this study, it might be possible to precipitate glycosides selectively from crude plant extracts. The development of a simple purification of glycosides is in progress.

Summary

Cyclodexrin glucanotransferase (CGTase) from *Bacillus macerans* was found to synthesize not only mono and oligoglucosylated products but also polyglucosylated ones by transglycosylation from α-cyclodextrin (α-CD) to various glycosides, although other 3 CGTases from *B. stearothermophilus*, *B. circulans* and *B. megaterium* synthesized mono and oligoglucosylated ones mainly.

The synthesis of the polyglucosylated products from various glycosides including rubusoside, stevioside, geniposide and rutin glucoside were also studied.

CHAPTER II. Synthesis of Glycosides by Cultured Plant Cells

Section 1. Glucosylation of Vanillin by Cultured Plant Cells

Introduction

Glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. It allows conversion of water-insoluble compounds to water-soluble ones and improves pharmacological properties, as in the case of salicylic acid.[4] In recent years, it has been demonstrated that cultures of various plant cells glycosylate many kinds of exogenous compounds, such as phenolics,[49-53] steroids,[54,55] flavonoids,[56-58] and steviol.[59]

Vanillin, the major component of vanilla flavor, is the most widely used flavoring agent for sweet foods, such as confectioneries, desserts, and ice creams. Furthermore, vanillin has strong antimutagenic effects when mutagenesis is induced by 4-nitroquinoline 1-oxide (4NQO) in *Escherichia coli*,[60] and it also has antimicrobial activity against fungi and yeast.[61] However, it has not been used as an antimutagenic or antimicrobial agent in food manufacturing industries.

In this section, the author describe the improvement of the properties of vanillin, without any loss of antimutagenic and antimicrobial activity, and the possibility of glucosylation of vanillin for improvement of foods.

Materials and Methods

Cell culture. Six culture strains used in this study were derived from the following plant materials in the years indicated; *Coffea arabica* (leaf, 1988), *Gardenia jasminoides* (leaf, 1988), *Medicago sativa* (hypocotyl, 1989), *Nicotiana tabacum* (leaf, 1989), *Theohroma cacao* (leaf, 1987), and *Prunus amygdalus* (hypocotyl, 1987). All suspension cultures were maintained in modified Murashige and Skoog's (MS) medium that contained per liter: 475 mg KNO_3, 412.5 mg NH_4NO_3, 110 mg $CaCl_2 \cdot 2H_2O$, 92.5 mg $MgSO_4 \cdot 7H_2O$, 85 mg KH_2PO_4, 21 mg Fe-Na-EDTA, 11.2 mg $MnSO_4 \cdot 7H_2O$, 4.3 mg $ZnSO_4 \cdot 7H_2O$, 3.1 mg H_3BO_3, 0.125 mg $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg $CuSO_4 \cdot 5H_2O$, 100 mg myo-inositol, 10 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, and 30 g sucrose (adjusted to pH 5.7 before autoclaving). The medium had been added 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) for *C. arabica*, *G. jasminoides*, and *M. sativa*; 1 μM 2,4-D for *N. tabacum*; 5 μM isopentenyladenine (2iP) and 0.5 μM indole-3-butyric acid (IBA) for *T. cacao*; and 5 μM benzyladenine (BA) and 5 μM IBA for *P. amygdalus*. Cultures were agitated in 300-ml conical flasks containing 100 ml of medium on a rotary shaker at 100 strokes/min at 25 °C in the dark, and cells were subcultured at two-week intervals.

Glucosylation experiments. In glucosylation experiments, cultured cells (1.5-2.0 ml packed cell volume) were inoculated into a 50-ml conical flask that contained 10 ml of the medium described above and incubated under the same conditions. After a two-week culture (stationary phase), the cultured

cells (2 ml packed cell volume) were transferred to fresh medium that had been mixed aseptically with vanillin (1 mM as a final concentration) and their culture was continued.

Quantitative assay. After culture with vanillin for 24 h, the harvested cells (1 ml as packed cell volume) were homogenized. The glucoside in the supernatant (500 μl) was hydrolyzed with 10 units/ml of β-glucosidase (from almonds, Sigma Chemical Co., Ltd.) at 37 °C and pH 5.5 for 16 h. The amount of aglycone in the hydrolysate was measured as vanillin by HPLC on an ODS column (E. Merck) that was eluted with methanol-H_2O (25:75, v/v) at a flow rate of 0.5 ml/min at 60 °C with detection by absorbance at 270 nm. The residual vanillin before β-glucosidase treatment was also measured by HPLC on an ODS as a blank. In this system, the retention time of vanillin was approximately 15.7 min.

The glucoside formed was measured as vanillin that was detected by HPLC and the efficiency of glucosylation was expressed as the percentage of vanillin that had been glucosylated by the cultured cells. All values represented the means of results from three independent experiments.

Extraction and purification of the glucoside. Harvested cells (200 ml packed cell volume) were homogenized in the culture medium. The homogenate was ultrafiltered and the fraction of less than 10 kDa in molecular size was put on a polyamide column, then chromatographed on an anion-exchange column (Diaion SA12; Mitsubishi Kasei). The flow-through fraction

was put on a charcoal column and eluted with 100% methanol. The eluate was concentrated and then fractionated by chromatography on a silica gel column that was eluted with *n*-butanol-acetic acid-H_2O (4:1:1, v/v). The active fraction was purified by preparative HPLC on an ODS column that was eluted with methanol-H_2O (35:65, v/v) at a flow rate of 7.0 ml/min at room temperature. The purified glucoside (7 mg) was obtained as a white powder by lyophilization.

Glucosidase treatments. To analyze its structure, the purified glucoside was hydrolyzed by 10 units/ml of α-glucosidase (from yeast, Seikagaku Corporation) or β-glucosidase (from almonds) at 37 °C for 16 h. After hydrolysis, the released glucose was measured by the glucose oxidase method,[38] and the released vanillin was measured by HPLC on an ODS column, as described above.

FAB-MS, ^{1}H-NMR and ^{13}C-NMR. FAB-MS were obtained with a JMS-DX303 system (JEOL) with a direct inlet system. NMR spectra were recorded with a JEOL GSX-400 (400 MHz) system (JEOL) in CD_3OD, with tetramethylsilane (TMS) as the internal reference. ^{1}H-NMR: δ 9.84 (1H, s, CHO); 7.51 (2H, d, aromatic H); 7.33 (1H, d, aromatic H); 5.07 (1H, d, anomeric H-1'); 3.91 (1H, d, H-6'a); 3.87 (1H, d, H-6'b); 3.84 (3H, s, OCH_3); 3.38-3.70 (4H, m). For the results of ^{13}C-NMR, see Table III.

Antimutagenic activity. *Salmonella typhimurium* TA98 and TA100 were cultured on Difco nutrient broth overnight, and then the cells were washed three times by centrifugation. They were suspended in 66 mM phosphate buffer (pH 6.8) at a final concentration of 1×10^9 cells/ml and then treated with 2 μg/ml 4-nitroquinoline 1-oxide (4NQO) at 37 °C for 15 min. The treated cells were washed to remove the mutagen and resuspended in the same phosphate buffer.

For His^+ reverse mutation assays, semi-enriched minimal (SEM) agar medium[62] was used, because both surviving colonies and His^+ revertant colonies can be detected on SEM plates. The SEM agar medium consisted of Vogel Bonner E medium (10 g K_2HPO_4, 3.5 g $Na(NH_4)HPO_4\cdot4H_2O$, 2.0 g citric acid monohydrate, and 0.2 g $MgSO_4\cdot7H_2O$ per liter) with 0.4% glucose, 1.2% Difco agar, 5% liquid nutrient broth (0.8% Difco nutrient broth powder and 0.5% NaCl), and 10 μg/ml casein hydrolysate.

To count His^+ revertants, 1-ml portions of the treated suspension of the cells and 0.1 ml of each test compound, dissolved in methanol at several concentrations, were incubated at 37 °C for 30 min. The treated cells were collected by centrifugation, and added to 2 ml of top agar (0.6% agar and 0.6% NaCl) and then poured onto SEM agar plates.

To count viable cells, 0.1-ml portions of a diluted suspension of the cells (1×10^{-6} -fold dilution) were added to 2 ml of top agar (0.45% agar and 0.45% NaCl) and then poured onto SEM agar plates. After incubation at 37 °C for 2 days, His^+ revertants and viable cells were counted. The antimutagenic activity (N) was expressed as the number of induced His^+ revertants per 10^7 viable cells.

Antimicrobial activity. Assays for antimicrobial activity were done on potato medium (200 g of potato infusion and 20 g of glucose per liter) for *Aspergillus niger* and *A. flavous*, on YM medium (5 g of peptone, 3 g of yeast extract, 3 g of malt extract, and 10 g of glucose per liter) for *Zygosaccharomyces rouxii*, and on L medium (10 g of peptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose per liter) for *E. coli*. Each medium contained vanillin or its glucoside and cultures were incubated at 30 °C for fungi, at 25 °C for yeast, and at 37 °C for bacteria. Before use, each compound to be tested was dissolved in methanol for sterilization, then it was added to sterile culture medium at a specific concentration. The antimicrobial activity of each compound was estimated from the lowest concentration of the compound that inhibited growth of the microorganisms on each medium, which was taken as the minimum inhibitory concentration. Growth was monitored macroscopically.

Results

Glucosylation of vanillin in cultures of various plant cells

Six different lines of cultured cells, from *C. arabica*, *P. amygdalus*, *G. jasminoides*, *N. tabacum*, *T. cacao*, and *M. sativa*, were tested for their ability to glucosylate vanillin. As shown in Table I, the efficiency of glucosylation by *C. arabica* cells was highest, being more than 80% for a 24-h culture and the efficiency of conversion by *P. amygdalus*, *G. jasminoides*, and *N. tabacum* cells varied from 10-30%, for 24-, 36-, and 36-h cultures, respectively. Cells of *T. cacao* and *M. sativa* accumulated almost no glucoside during two weeks of culture. This experiment showed that various cultured cells can glucosylate a foreign compound, vanillin, which is not originally present in the cultured cells. However, the amount of vanillin glucosylated varied among the cultures.

In this study, because of their high glucosylation ability, the author used suspension cultures of *C. arabica* cells for glucosylation of vanillin.

Table I. Glucosylation of Vanillin by Cultures of Various Plant Cells

Plant cell	Efficiency of glucosylation (%)
C. arabica	84.5
P. amygdalus	30.2
G. jasminoides	16.3
N. tabacum	9.6
T. cacao	4.3
M. sativa	1.2

Each plant cell culture was cultured on the medium described in Materials and Methods. *C. arabica* cells and *P. amygdalus* cells were cultured for 24 h and other cells were cultured for 36 h.

Concentration of vanillin and the effciency of glucosylation

The effects of the concentration of vanillin on its glucosylation by *C. arabica* cells after incubation for 24 h are shown in Fig. 1. The maximum efficiency of glucosylation reached more than 80% at 1 mM vanillin and decreased at higher concentrations. Since excessive vanillin is toxic to cells, 1 mM vanillin seems to be the critical concentration at which the cellular capacity for glucosylation and the toxicity of vanillin are balanced.

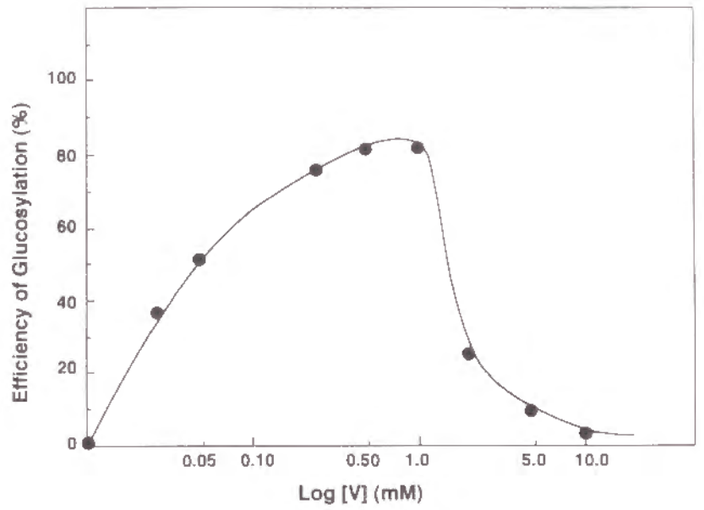


Fig. 1. Effects of the Concentration of Vanillin on Glucosylation by *C. arabica* Cells.

The concentrations of vanillin ([V]) applied to the cell cultures are expressed on a logarithmic scale. Cells were harvested 24 h after administration of vanillin. The vanillin glucoside was measured by HPLC.

Course of glucoside formation

The course of glucosylating vanillin was investigated. One mmol vanillin was added to *C. arabica* cells at the beginning of the culture. As shown in Fig.

2, suspension cultures of *C. arabica* cells converted vanillin into its glucoside, which accumulated in the cells. Only small quantities were released into the medium. When 1 mmol vanillin was administered, conversion into the glucoside was observed within 4 h after administration. More than 80% of the vanillin added to the medium was glucosylated within 24 h but it gradually decreased to 10% by the end of the growth cycle (two weeks of culture).

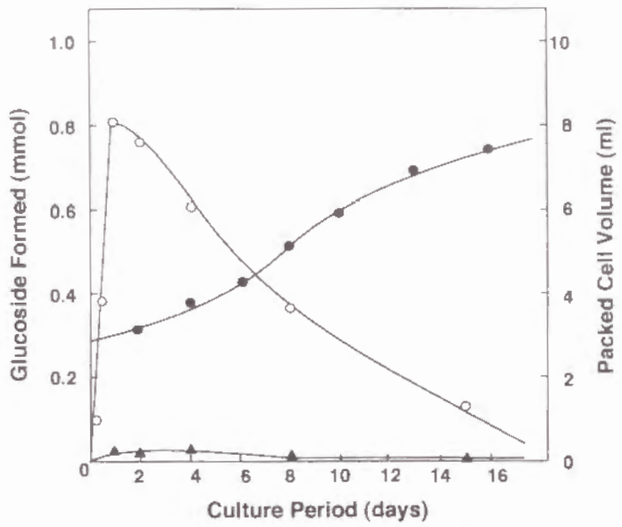


Fig. 2. Course of Glucosylation by *C. arabica* Cells.
(●), cell growth (ml packed cell volume); (○), the amount of glucoside in the cells (mmol); (▲), the amount of glucoside in the medium (mmol).

Optimum conditions for glucosylation

To examine the effects of phytohormones in the medium, suspension cultures, which had been subcultured in a medium containing 5 μM 2,4-D every two weeks, were transferred to media containing various concentrations of auxins and/or cytokinins and cultured for eight weeks. The cells (1.6 ml packed cell volume) were then transferred to the fresh medium containing 1 mM vanillin and cultured for 24 h. As shown in Table II, the amount of glucoside formed per ml packed cell volume was highest in medium with 5 μM 2,4-D and 5 μM kinetin (Kin), while cell growth was highest in medium with 5 μM 2,4-D and 0.5 μM Kin after two weeks in culture. Since the yield of the glucoside was based on both the amount of glucoside per ml packed cell volume and cell growth, the amount of glucoside per flask was highest in the medium (10 ml) with 5 μM 2,4-D and 0.5 μM Kin.

Table II. Effects of Phytohormones on Glucosylation of Vanillin by *C. arabica* Cells

Phytohormone	Cell Growth (ml PCV[*1)]/flask)	Glucoside Formed (mM/ml PCV)	Glucoside Formed (mM/flask)
5 μM IAA	1.33 ± 0.18[*2)]	0.82 ± 0.10	1.09 ± 0.20
5 μM IBA	2.03 ± 0.15	0.86 ± 0.03	1.75 ± 0.14
0.5 μM 2,4-D	3.57 ± 0.15	0.85 ± 0.44	3.04 ± 0.20
5 μM 2,4-D	4.80 ± 0.20	0.67 ± 0.04	3.21 ± 0.23
50 μM 2,4-D	1.93 ± 0.18	0.58 ± 0.02	1.12 ± 0.11
5 μM 2,4-D + 0.5 μM K	5.00 ± 0.31	0.85 ± 0.09	4.25 ± 0.51
5 μM 2,4-D + 5 μM K	3.80 ± 0.12	0.99 ± 0.14	3.78 ± 0.56

*1); packed cell volume *2); standard error

Characterization of the glucoside

1. Structure. Vanillin (152 mg) was added to a culture of *C. arabica* cells (200 ml packed cell volume) in modified MS medium with 5 μM 2,4-D and 0.5 μM Kin. After 24 h, the cells were homogenized in the medium and the

supernatant was ultrafiltered. The glucoside-containing fraction was purified by chromatography on a polyamide column, an anion-exchange column, a charcoal column, a silica gel column, and on an ODS column by preparative HPLC. The purified glucoside (7 mg) was obtained as a white powder.

After hydrolysis by β-glucosidase, the glucoside (0.120 mM) yielded vanillin (0.112 mM) as the aglycone, and the corresponding amount of glucose (0.120 mM). The molar ratio of vanillin to glucose was 0.93. α-Glucosidase did not hydrolyze the glucoside. The results suggested that the glucoside was vanillin-β-D-monoglucopyranoside.

FAB-MS gave a molecular ion at m/z 337 ($[M + Na]^+$). The fragment peak at m/z 175 ($[(M + Na) - 162]^+$) was due to the subsequent loss of the glucosyl unit.

In the ^{13}C-NMR spectrum (Table III), 14 carbon signals were observed; 8 carbon signals, δ 153.56-111.83 (aromatic group), 56.67 (methoxyl), and 192.99 (aldehyde), were assignable to the vanillin moiety and the remainder, δ 101.85-62.50, to glucose. The observation of the signal of the anomeric carbon at δ 101.85 suggests the β configuration.

In the ^{1}H-NMR spectrum (see Materials and Methods), signals assignable to the aromatic protons were observed at δ 7.56-7.31, and those assignable to methoxyl and aldehyde groups were observed at δ 3.84 and 9.84, respectively. A signal at δ 5.07, assignable to anomeric proton of glucose, was also observed. The large coupling constant (J= 7.3 Hz) of the anomeric proton in the glucoside also suggests the β configuration for the anomeric center.

Table III. ^{13}C-NMR Spectral Data for Glucosylvanillin Expressed as δ Values (ppm form the Internal Standard TMS in CD_3OD)

C-aromatic	δ(ppm)
1	153.6
2	151.3
3	116.6
4	127.0
5	132.9
6	111.8
C-Glu	
1	101.9
2	77.9
3	77.8
4	75.2
5	71.3
6	62.5
C-Me	56.7
C-CHO	193.0

Consequently, the glucoside can be identified as 4-formyl-2-methoxyphenyl-*O*-β-D-glucopyranoside, namely glucosylvanillin.

2. Antimutagenic activity.

The antimutagenic activities of glucosylvanillin and vanillin are shown in Fig. 3. Both compounds had almost the same effect on mutagenesis. An obvious reduction in the rates of induced mutation was observed in the presence of either compound at 10 μmol/plate.

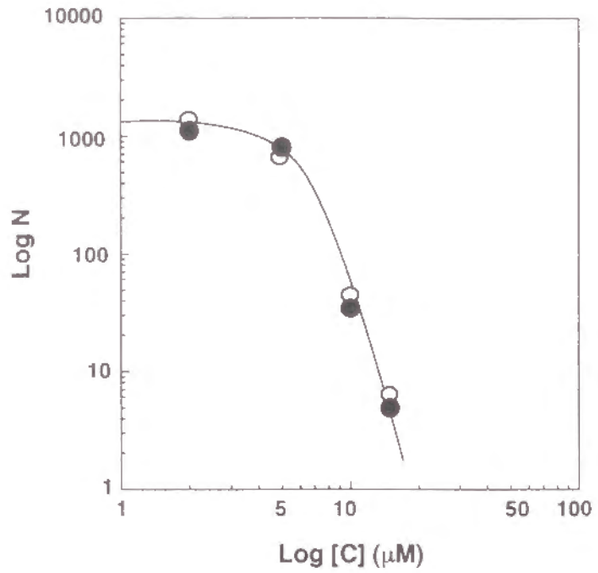


Fig. 3. Antimutagenic Activities of Vanillin and Glucosylvanillin.
The antimutagenic activities *versus* the concentration of vanillin or glucosylvanillin are expressed on a logarithmic scale. N, the antimutagenic activities with *Salmonella typhimurium*; [C], concentration of vanillin or glucosylvanillin (μM). (○) vanillin; (●), glucosylvanillin. All values are averages from two plates. The values for TA100 were almost as same as those for TA98. The test compounds were assayed at concentrations of 0, 2, 10, and 20 μmol in 1.1-ml reaction mixtures.

3. Antimicrobial activity.

The antimicrobial activity of glucosylvanillin against *A. niger*, *A. flavus*, *Z. rouxii*, and *E. coli* is shown in Table IV. The organisms grew well in both vanillin-free and glucosylvanillin-free media, but growth inhibition was observed in the presence of more than 100 ppm glucosylvanillin for *A. niger*, *A. flavus*, and *Z. rouxii*. A high concentration of glucosylvanillin (1000 ppm) was required for the complete inhibition of their growth. The growth of *E. coli* was not inhibited even at 2000 ppm of glucosylvanillin, the highest concentration tested. Vanillin was found to have similar effects as glucosylvanillin (data not shown).

Table IV. Antimicrobial Activities of Glucosylvanillin

Concentration of GV [*1)] (ppm)	Microorganism			
	A. niger	*A. flavus*	*Z. rouxii*	*E. coli*
Control	–[*2)]	–	–	–
100	+	+	+	–
200	++	++	++	–
1000	+++	+++	+++	–
2000	+++	+++	+++	–

*1); glucosylvanillin *2); – ; Cells grew well ++ ; Cells barely grew
+ ; Cells grew somewhat +++ ; Cells failed to grow

Discussion

Glycosylation is a characteristic transformation in plant cells. It has been tried to improve the chemical properties and physiological functions of industrially and pharmacologically useful compounds.[4] There are three reasons for application of plant cells. The first is that glucosylation by cultured plant cells is a one-step reaction, on the other hand, chemical synthesis is a multiple-step reaction consisting acetylation, glucosylation, and deacetylation. The second is that glucosylation by cultured plant cells is position-specific, that is, at the β-linkage at C-1 of the glucose moiety. The third is that there is little information on microbial enzymes that glucosylate compounds having phenolic OH groups such as vanillin.

There have been many studies of glycosylation using cultures of various plant cells.[53,59,63] In this section, six different plant cell cultures were tested for their abilities to glucosylate vanillin. Large differences in glucosylation ability were found among these cultured cells. This suggests the necessity of selecting cells that glucosylate an administered compound at a high rate. These experiments showed that the optimum conditions for glucosylating vanillin were 1 mM vanillin to be applied to cultures of *C. arabica* cells in modified MS medium with 5 μM 2,4-D and 0.5 μM Kin. More than 80% of the added vanillin was glucosylated within 24 h after its administration. Compared with other studies, the efficiency of glucosylating vanillin by *C. arabica* cells was as high as that of hydroquinone by *Datura innoxia*[49,53] and it was much higher than other results, such as esculetin (43% as efficiency of glucosylation) by *Perilla frutescens*[53] and quercetin (31 %) by *Vitis* sp.[56] As *C. arabica* cells

had such a superior glucosylating activity, they were suitable for the purposes of this study. This study also demonstrated that the conversion product, glucosylvanillin, maintained both the antimutagenic and the antimicrobial activities of vanillin, and that these activities of glucosylvanillin were the same as those of vanillin. It has been reported that vanillin acts as an antimutagen in mammalian cells both *in vitro* and *in vivo*, for example, it suppresses mutations induced by mitomycin C,[64] by UV light, and by X-rays.[65] It also has an antimutagenic effect in the mouse spot test which is a method for detecting somatic cell mutations *in vivo*.[65] It is of interest that a glucosylated compound such as glucosylvanillin maintains the activity of the compound with respect to prevention of mutation and inhibition of the growth of microorganisms.

Vanillin has a specific flavor that is most in demand. However, because of this, it cannot be used at the high concentrations at which it has its biological activities. Glucosylvanillin was found to have no specific flavor, as it was evaluated by a human sensory panel (five professional flavorists). Glucosylation converted the volatile compound vanillin into non-volatile, more hydrophilic glucosylvanillin. Therefore, glucosylvanillin may be of great value as an edible compound with many biological activities. It might be used as a new ingredient for functional foods, although further efforts are needed to produce it on a large scale.

Summary

Vanillin was converted into the corresponding glucoside in suspension-cultured cells of *Coffea arabica*. The maximum efficiency of glucosylation was 85% within 24h after the addition of 1 mM vanillin when cultured in a modified Murashige and Skoog's medium with 5 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM kinetin. The glucoside was identified as 4-formyl-2-methoxyphenyl-α-D-glucopyranoside by ^{1}H-NMR, ^{13}C-NMR, FAB-MS, and hydrolysis by α- and β-glucosidases. It retained the antimutagenic and antimicrobial activities of vanillin.

Section 2. Glucosylation of Capsaicin by Cultured Plant Cells

Introduction

Capsaicin is a pungent principle of the hot pepper, which is used as an important spice for enhancing the palatability of food and medically as a counterirritant. The chemistry, biochemistry, and pharmacology of capsaicin have been reviewed.[66] The study of capsaicin has now been focused on its nutritional benefits, as well as its effects on the nervous system through substance P.[67,68] It was reported that capsaicin reduced the perirenal adipose tissue weight and serum triglyceride concentration in rats[69] by enhancing energy metabolism through a β-adrenergic action *in vivo*,[70] when fed to the rats together with a high fat diet. Furthermore, it was demonstrated that the enhancement of energy metabolism occurred through catecholamine secretion from the adrenal medulla[71,72] as the result of the activation of the central nervous system.[73] In humans, it was reported that the ingestion of chili sauce with meals resulted in a marked increase in energy metabolism.[74] In spite of such physiological functions, the use of capsaicin as a food ingredient has been limited, because of its strong pungency and low solubility.

Glycosylation may be considered a useful method for improving some characteristics of compounds having biological activities. It allows conversion of a water-insoluble compound to a water-soluble one to improve its pharmacological properties, as reported for salicylic acid.[4] It has been demonstrated that various plant cell cultures are capable of glycosylating

several kinds of exogenously administered compounds, including salicylic acid (*Mallotus japonicus*),[50] *o*-, *m*-, and *p*-hydroxybenzoic acid (*M. japonicus*),[52] steviol (*Eucalyptus perriniana* and *Coffea arabica*),[59] and eugenol and isoeugenol (*E. perriniana*).[75]

This section describes possible conversion of capsaicin to a food ingredient having biological activities by glucosylation, using plant cell cultures.

Materials and methods

Cell cultures. Six different lines of cell cultures, from *C. arabica*, *Gardenia jasminoides*, *Nicotiana tabacum*, *Prunus amygdalus*, *Theobroma cacao*, and *Capsicum annuum*, were tested for their ability to glucosylate capsaicin. All suspension cultures have been maintained in a modified Murashige and Skoog's (MS) medium with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin (Kin) (adjusted to pH 5.7 before autoclaving) for *C. arabica* and *G. jasminoides*; with 1 μM 2,4-D for *N. tabacum*; with 5 μM benzyladenine (BA) and 5 μM indole-3-butyric acid (IBA) for *P. amygdalus*; and with 5 μM isopentenyladenine (2iP) and 0.5 μM IBA for *T. cacao* (described in Chapter II, section 1). Suspension cultures of *C. annuum* was newly derived from hypocotyl in 1992, and maintained in a modified MS medium with 5 μM 2,4-D and 0.5 μM Kin. Cultures were agitated in 300-ml conical flasks containing 100 ml of medium on a rotary shaker at 100 strokes/min at 25 °C in the dark, and cells were subcultured at two-week intervals.

Glucosylation experiments. In glucosylation experiments, cultured cells (20 ml packed cell volume) were inoculated into a 500-ml conical flasks had contained 100 ml of the medium described above and incubated at the same conditions. After two-week culture (stationary phase), the cultured cells (20 ml packed cell volume) were transferred to fresh medium that had been supplemented aseptically with capsaicin (0.5 mM as a final concentration) and their cultures were continued.

Quantitative assay. After culturing with capsaicin for 48 h, the harvested cells were homogenized. The glucoside in the supernatant was hydrolyzed with 10 units of β-glucosidase (from almonds, Sigma Chemical Co., Ltd.) at 37 °C and pH 5.5 for 16 h. The amount of glucoside formed was measured as capsaicin (the aglycone in the hydrolysate) by HPLC on an ODS column (E. Merck) eluted with 50 mM KH_2PO_4-acetonitrile (1:1, v/v) at a flow rate of 0.7 ml/min at 40 °C detecting absorbance at 280 nm. The residual capsaicin before β-glucosidase treatment was also measured by HPLC on an ODS as a blank. All values represented the means of results from two independent experiments.

Extraction and purification of the glucoside. Harvested cells (200 ml packed cell volume) were separated from culture medium and lyophilized. The lyophilized powder was dissolved in 10 volume (w/v) of H_2O and extracted with the same volume of ethyl acetate. The extract was concentrated *in vacuo* and the capsaicin glucoside was purified with a preparative HPLC on an ODS column (E. Merck) that was eluted with 50 mM KH_2PO_4-acetonitrile (1:1, v/v) at a flow rate of 7.0 ml/min at room temperature by detecting absorbance at 280 nm. The purified glucoside (7 mg) was obtained as a white powder by lyophilization.

Glucosidase treatments. In order to elucidate the structure of the glucoside, the purified glucoside (0.5 mg/ml) was hydrolyzed by treatment of 10 units/ml of α-glucosidase (from yeast, Seikagaku Corporation) and β-glucosidase (from almonds) at 37 °C for 16 h. After hydrolysis, the released

glucose was quantitated by glucose oxidase method,[38] and the released capsaisin was quantitated by HPLC on an ODS column, described above.

FAB-MS and ^{1}H-NMR. FAB-MS was done with JMS-DX303 system (JEOL) with a direct inlet system, and ^{1}H-NMR spectra were recorded with GSX-400 (400 MHz) system (JEOL) in CD_3OD with tetramethylsilane (TMS) as the internal reference. The ^{1}H-NMR spectrum of the glucoside was as follows; δ 0.955 (6H, d, CH_3), 1.36 (2H, p, CH_2), 1.62 (2H, p, CH_2), 1.98 (2H, q, CH_2C=C), 2.18 (2H, t, CH_2C=O), 2.32 (1H, m, CH), 3.30-3.48 (4H, m), 3.66 (1H, dd, H-6'a), 3.69 (1H, dd, H-6'b), 3.84 (3H, s, OCH_3), 4.30 (2H, d, aromatic CH_2), 4.84 (1H, br, NH), 5.36 (1H, d, anomeric H: J = 6.6 Hz), 5.37 (2H, m, HC=CH), 6.82 (1H, d, 6-aromatic H), 6.94 (1H, s, 2-aromatic H), 7.11 (1H, d, 5-aromatic H).

Sensory evaluation. The samples were dissolved in distilled water and diluted stepwise in tenths to a final concentration of 10^{-8} M. The tests, which started from a 10^{-8} M solution, were continued until the six panelists could detect the taste of the sample. The lowest detectable concentration was defined as a threshold value.

Results

Glucosylation of capsaicin by cultured plant cells

Among six lines of cell cultures such as *C. arabica*, *G. jasminoides*, *N. tabacum*, *P. amygdalus*, *T. cacao*, and *C. annuum*, only *C. arabica* cells could glucosylate capsaicin; the other five cultures could not accumulate glucoside during two weeks of culture. *C. arabica* cells yielded 15.8 mg of capsaicin glucoside in the cells for a 48-h culture, when 153 mg of capsaicin was administered to the culture (containing 280 ml of packed cell volume/liter of a modified MS medium with 5 μM 2,4-D and 0.5 μM Kin) (Fig. 1).

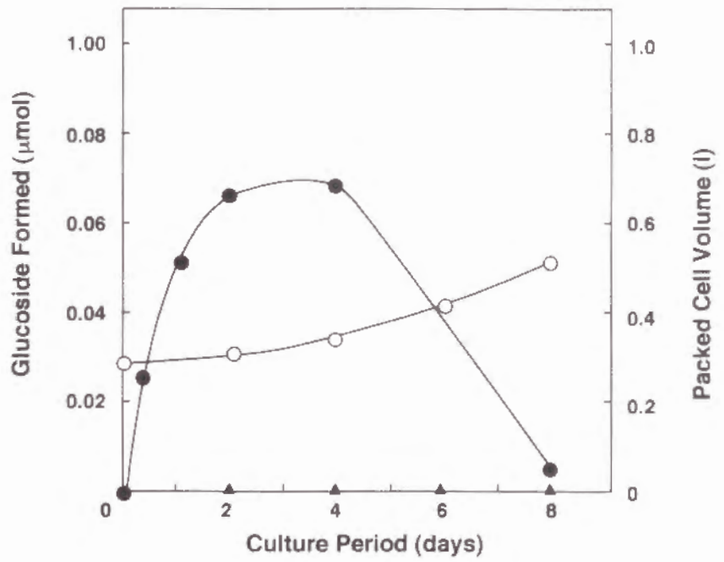


Fig. 1. Course of Glucosylation of Capsaicin by *C. arabica* Cells.
(○), cell growth (liters of packed cell volume); (●), the amount of glucoside in the cells (μmol); (▲), the amount of glucoside in the medium (μmol).

Purification of the glucoside

The glucoside was purified from the harvested cells, which cultured with 153 mg capsaicin. The lyophilized *C. arabica* cells were extracted with ethyl acetate, and the glucoside formed was purified with a preparative HPLC with an ODS column. Seven mg of purified glucoside was obtained as a white powder by lyophilization.

Structure of the glucoside

To analyze the structure of the glucoside, α- and β-glucosidase treatments, FAB-MS, and ^{1}H-NMR were done. The glucoside (0.5 mg/ml) was hydrolyzed by treatment of 10 units/ml of α-glucosidase (from yeast) or β-glucosidase (from almonds) at 37 °C for 16 h. Then the released capsaicin was measured by HPLC described above. The released glucose was measured by the glucose oxidase method. By hydrolysis with β-glucosidase, the glucoside yielded 0.93 mM capsaicin (aglycone) and the corresponding amount of glucose (Table I). The molar ratio of capsaicin to glucose was 0.86. α-Glucosidase did not hydrolyze the glucoside.

Table I. Enzymatic Analysis of Capsaicin Glucoside

Treatment	Released Cp* (mM)	Released Glucose (mM)	Ratio (Cp/Glucose)
α-Glucosidase	ND**	ND	-
β-Glucosidase	930	1080	0.86

*, Cp; capsaicin **, ND; not detected

In addition, the FAB-MS spectrum of the glucoside gave a molecular ion at m/z 490 [M + Na]. The fragment peak at m/z 328 [M + Na - 162] was due to the loss of a glucosyl unit. In the ^{1}H-NMR spectrum of it (see, Materials and Methods), the signals assignable to the aromatic protons were observed at δ 7.11-6.82, to carbon chain moiety at δ 5.37-4.30 and δ 2.32-0.955, to methoxy group at δ 3.84 and to glucose moiety at δ 3.69-3.30, respectively. The signal at δ 5.36 assignable to anomeric protons of glucose were also observed. A large coupling constant (J = 6.6 Hz) of the anomeric proton in the glucoside suggested the β configuration for the anomeric center.

Therefore, the glucoside could be identified as capsaicin-β-D-glucopyranoside (Fig. 2).

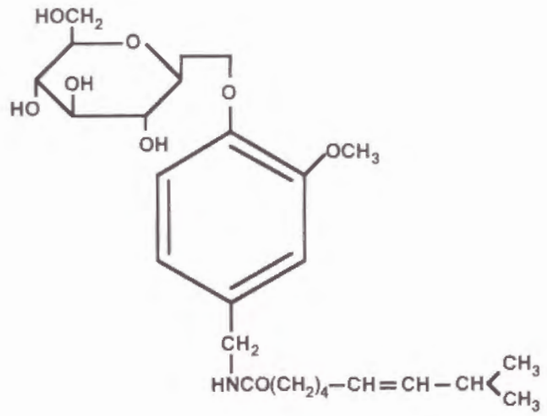


Fig. 2. Structure of Capsaicin-β-D-glucopyranoside.

Sensory evaluation

The intensity of pungency of capsaicin glucoside was estimated by comparison with that of capsaicin by a panel of six professional tasters. As shown in Table II, the threshold value of capsaicin glucoside was 10^{-4} M, but that of capsaicin was 10^{-6} M suggesting that the former was approximately 100 times less pungent than the latter.

Table II. Sensory Test of Capsaicin (Cp) and Capsaicin Glucoside (Cp-G)

	Concentration (M)					
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Cp	++++	+++	+	±	–	–
Cp-G	+	±	–	–	NM*)	NM

NM*); not measured

Discussion

In this section, capsaicin was glucosylated by *C. arabica* cells and its structure and pungency were determined. The glucosylation of the OH group of the aromatic ring found to lead to significant loss of activity, approximately 100 times lower than that of capsaicin. According to Szolcsányi and Jancsó-Gábor,[76] capsaicin is a structurally specific compound having several critical moieties for binding to the receptor molecule. The result in this study suggests the importance of the phenolic OH group for the pungent property. A decrease of the intensity as well as an improvement in the quality of taste has been reported in the enzymatic transglycosylation of stevioside and rubusoside by cyclodextrin glucanotransferase.[5,27] These results suggest that glucosylation may convert certain substrates into compounds with more desirable physiological activities.

In this study on capsaicin, glucosylation of the phenolic OH group resulted in a remarkable loss of pungency as well as an increase in water-solubility, making it possible to eat glucosylated capsaicin easily at the physiologically active level. If the yield of capsaicin glucoside could be improved, this compound would be promising for practical use as a unique food ingredient with biological activities.

Summary

Capsaicin was converted into the corresponding glucoside when administered to cell suspension cultures of *Coffea arabica* cultured in a modified Murashige and Skoog's medium with 5 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM kinetin.

The glucoside was identified as capsaicin-β-D-glucopyranoside by FAB-MS, ^{1}H-NMR, and hydrolysis with α- and β-glucosidases. The pungency of the glucoside was approximately 1/100 of that of capsaicin.

Section 3. Synthesis of 3,4-Dimethoxyphenyl-*O*-β-D-glucopyranoside and its Related Glycosides by Cultured Plant Cells

Introduction

3,4-Dimethoxyphenyl-*O*-β-D-glucopyranoside (3,4-DMP glucoside) was purified from non-sugar fraction of crude black sugar prepared from sugar cane,[77] and has the activity that reduced the level of plasma insulin without elevating plasma glucose in the glucose tolerance test, suggesting that it might inhibit the absorption of glucose from the small intestines.[78] It is expected that the glucoside protects pathological changes induced by taking a large amount of refined sugar, such as hyperlipemia, obesity, diabetes and arteriosclerosis. However, as there is only a little quantity of 3,4-DMP glucoside in sugar cane, it has not been applied to medicines or food materials, etc.

Glycosylation is considered to be an important method for the synthesis and the structural modification of compounds with useful biological activities in the case of salicylic acid.[4] And recently, it has been demonstrated that the cultures of various plant cells have the ability to glycosylate many kinds of exogenously administered compounds, such as phenolics,[49,51-53] steroids,[54,55] flavonoids,[56-58] and steviol.[59]

In this section, the author described to synthesize a large amount of 3,4-DMP glucoside by glucosylation of 3,4-DMP, and to synthesize another six kinds of glucosides from seven kinds of methoxyphenol derivatives using cultured plant cells.

Materials and Methods

Materials. *o*-Methoxyphenol (*o*-MMP), *m*-methoxyphenol (*m*-MMP), *p*-methoxyphenol (*p*-MMP), 2,3-dimethoxyphenol (2,3-DMP), 2,6-dimethoxyphenol (2,6-DMP), 3,4-dimethoxyphenol (3,4-DMP), 3,5-dimethoxyphenol (3,5-DMP), 3,4,5-trimethoxyphenol (3,4,5-TMP) were purchased from Sigma Chemical Co., Ltd.

Cell culture. Five culture strains used in this study were derived from the following plant materials in the years indicated, and were maintained in modified Murashige and Skoog's (MS) medium supplemented with; 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 μM kinetin (Kin) (adjusted to pH 5.7 before autoclaving) for *Coffea arabica* (leaf, 1988), *Dianthus caryophyllus* (hypocotyl, 1991), *Gardenia jasminoides* (leaf, 1988) and *Lupinus polyphyllus* (hypocotyl, 1991) and with 5 μM isopentenyladenine (2iP) and 0.5 μM indole-3-butyric acid (IBA) for *Theobroma cacao* (leaf, 1987) (described in Chapter II, section 1). Cultures were agitated in 300-ml conical flasks had contained 100 ml of the medium on a rotary shaker operated at 100 strokes/min at 25 °C in the dark, and the cells were subcultured at two-week intervals.

Glucosylation experiments. In glucosylation experiments, cultured cells (1 ml packed cell volume) were inoculated into a 50-ml conical flask had contained 10 ml of the medium described above and incubated under the same conditions. After two-week culture (stationary phase), the cultured cells (2 ml packed cell volume) were transferred to fresh medium that had been

supplemented aseptically with each methoxyphenol derivative (1 mM as a final concentration) and their cultures were continued.

Quantitative assay. After culturing with each methoxyphenol derivative for 48 h, the harvested cells (1 ml packed cell volume) were homogenated. The formed glucoside in the supernatant (500 μl) was hydrolyzed with 10 units/ml of β-glucosidase (from almonds, Sigma Chemical Co., Ltd.) at 40 °C and pH 5.5 for 16 h. The amount of aglycone in the hydrolysate was determined as each methoxyphenol derivative by HPLC on an ODS column (E. Merck) that was eluted with methanol-H_2O (25:75, v/v) at a flow rate of 0.5 ml/min at 60 °C detecting absorbance at 270 nm. The residual amount of each methoxyphenol derivative before β-glucosidase treatment was also determined by HPLC on an ODS as a blank.

The amount of glucoside formed was determined as each methoxyphenol detected by HPLC, and the efficiency of glucosylation was expressed as the percentage of each methoxyphenol derivative that had been glucosylated by cultured cells. All values represented the means of results from three independent experiments.

Extraction and purification of the glucoside. Harvested cells (200 ml packed cell volume) were washed with H_2O, then homogenized in the culture medium. The homogenate was boiled at 100 °C for 15 min. The supernatant was lyophilized and the glucoside was extracted with hot methanol. The extract was applied to a preparative HPLC on an ODS column that was

eluted with methanol-H_2O (35:65, v/v) at a flow rate of 7.0 ml/min at room temperature. Purified glucoside (79 mg) was obtained as a white powder by lyophilization.

Glucosidase treatment. In order to elucidate the structure of the glucoside, the purified glucoside was hydrolyzed by 10 units/ml of α-glucosidase (from yeast) or β-glucosidase (from almonds) at 40 °C for 16 h. After hydrolysis, the released glucose was measured by glucose oxidase method,[38] and the released 3,4-DMP was measured by HPLC on an ODS, as described above.

^{1}H-NMR. NMR spectra were recorded with a JNM-GX270 (270 MHz) system (JEOL) in CD_3OD with tetramethylsilane (TMS) as the internal reference.

Results

Glucosylation of 3,4-dimethoxyphenol in cultures of various plant cells Five different lines of cultured cells, from *C. arabica*, *D. caryoaphyllus*, *L. polyphyllus*, *T. cacao*, and *G. jasminoides*, were tested for the ability to glucosylate 3,4-DMP. As shown in Fig. 1, the efficiency of glucosylation of *C. arabica* cells was highest, being more than 40% for 96-h culture. Those of *D. caryophyllus*, *L. polyohyllus*, *T. cacao*, and *G. jasminoides* cells were less than 15% for 48-h culture, and they could not accnmulate the glucosides any more during 2 week-culture.

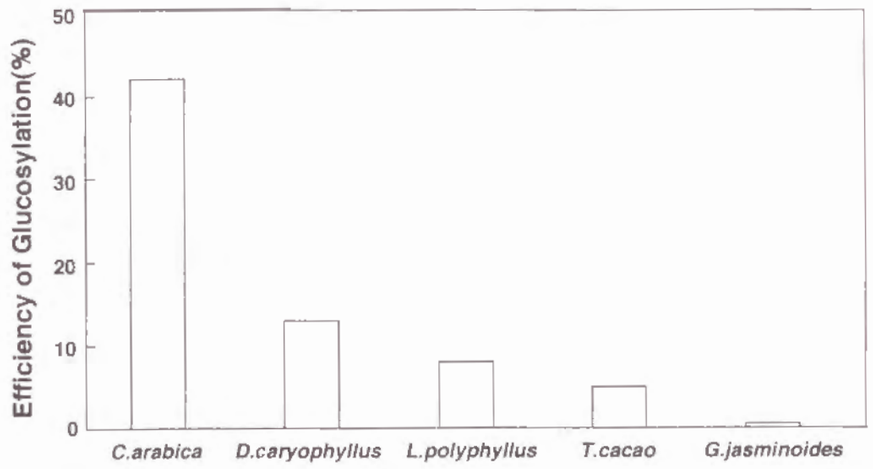


Fig. 1. Glucosylation of 3,4-Dimethoxyphenol by Cultures of Various Plant Cells.

Each plant cells was cultured on the medium described in Materials and Methods. The efficiencies of glucosylation were measured at 96 h-culture of *C. arabica* cells and at 48 h-culture of other cells.

This experiment showed that various cultured cells can glucosylate foreign compounds, such as 3,4-DMP which is not originally present in the

cultured cells. However, the amount of glucosylated 3,4-DMP varies with the cultures.

In the present study, because of their high glucosylation ability, the author used suspension cultures of *C. arabica* for glucosylation of 3,4-DMP.

Concentration of 3,4-dimethoxyphenol and the efficiency of glucosylation

The effects of the concentration of 3,4-DMP on its glucosylation by *C. arabica* cells after culturing for 96 h are shown in Fig. 2. The maximum efficiency of glucosylation reached more than 40% at 1-2 mM 3,4-DMP and decreased at higher concentrations. Since excessive 3,4-DMP is toxic to the cells, 2 mM 3,4-DMP seems to be the critical concentration at which the cellular capacity for glucosylation and the toxicity of 3,4-DMP are balanced.

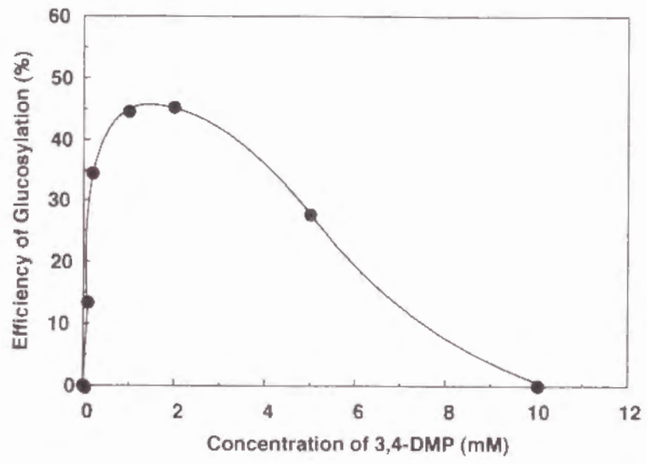


Fig. 2. Effects of Concentration of 3,4-Dimethoxyphenol on Glucosylation by *C. arabica* Cells.

Cells were harvested at 96 h after administration of 3,4-DMP, and 3,4-DMP glucoside was measured by HPLC.

Course of glucoside formation

The time course of glucosylating 3,4-DMP was investigated. One mM 3,4-DMP was added to *C. arabica* cells at the beginning of the culture. As shown in Fig. 3, the suspension cultures of *C. arabica* cells converted 3,4-DMP into its glucoside which accumulated in the cells. Only small quantities were released into the medium. When 1 mM 3,4-DMP was administered, conversion into the glucoside was observed at 10 h after administration. The extent of conversion reached more than 40% within 96 h and then decreased gradually.

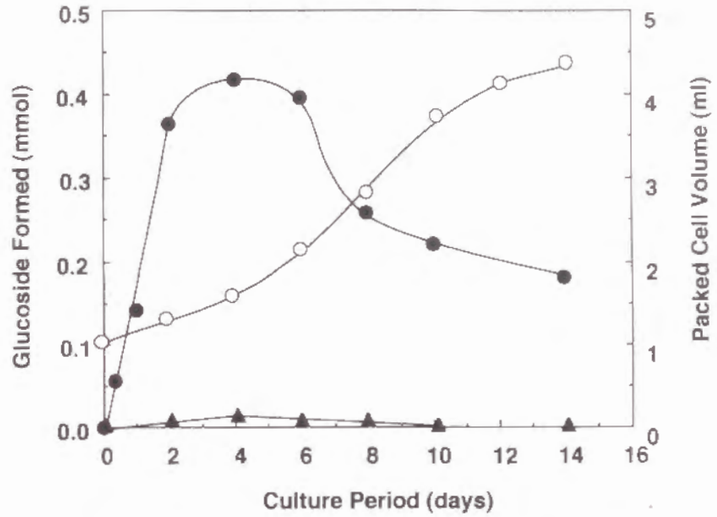


Fig. 3. Time Course of Glucosylation by *C. arabica* Cells.

(○), cell growth (ml packed cell volume); (●), the amount of glucoside in the cells (mmol); (▲), the amount of glucoside in the medium (mmol). Cells were harvested at various intervals after administration of 1 mM 3,4-DMP, and 3,4-DMP glucoside was measured by HPLC.

Optimum conditions for glucosylation

To examine the effects of phytohormones in the medium, *C. arabica* cells cultured in a medium containing various concentrations of auxins and/or cytokinins were used. The cells (1 ml packed cell volume) were transferred to the fresh medium containing 1 mM 3,4-DMP and cultured. As shown in Table I, among auxin, 2,4-D was the most effective on both cell growth and glucoside formation. In combination with 2,4-D and Kin, the amount of glucoside formed per ml packed cell volume was highest in medium with 5 μM 2,4-D and 0.5 μM Kin for 96-h culture, and cell growth was highest in the same medium after 2 week-culture.

Table I. Effects of Phytohormones on Glucosylation of 3,4-DMP by *C. arabica* Cells.

Phytohormones	Cell Growth (ml PCV*1)/Flask)	Glucoside Formed (mM/ml PCV)	Glucoside Formed (mM/Flask)
5 μM IAA	1.40±0.08*2)	4.18±0.28	5.86±0.69
5 μM IBA	1.60±1.83	2.24±0.25	3.59±0.57
5 μM NAA	1.83±0.12	9.96±3.03	18.27±6.41
0.5 μM 2,4-D	2.00±0.08	6.62±1.93	13.23±3.98
5 μM 2,4-D	4.03±0.12	17.30±0.52	69.77±1.06
50 μM 2,4-D	2.97±0.05	19.19±1.14	56.93±2.49
5 μM 2,4-D + 0.5 μM Kin	4.27±0.05	51.53±2.51	219.86±9.96
5 μM 2,4-D + 5 μM Kin	3.33±0.05	17.05±0.76	56.84±3.30

*1); Packed Cell Volume *2); Standard Error

Purification and structure of the glucoside

3,4-DMP (154 mg) was added to a culture of *C. arabica* cells (200 ml packed cell volume) in modified MS medium supplemented with 5 μM 2,4-D and 0.5 μM Kin. After 96 h, the cells were homogenized and boiled at 100 °C for 15 min. The supernatant was lyophilized and extracted with hot methanol. The extract was purified with a preparative HPLC on an ODS column. The purified glucoside (79 mg) was obtained as a white powder.

After hydrolysis by β-glucosidase, the glucoside (as a concentration of 11 mg/ml) yielded 3,4-DMP (35.0 mM) as aglycone, and the corresponding amount of glucose (33.4 mM). The molar ratio of 3,4-DMP to glucose was 1.05. α-Glucosidase did not hydrolyze the glucoside (Table II). The results suggested that the glucoside was 3,4-DMP-β-monoglucoside.

Table II. Enzymatic Analysis of 3,4-DMP Glucoside

Treatment	Released 3,4-DMP (mM)	Released Glucose (mM)	Ratio (3,4-DMP/Glucose)
α-Glucosidase	ND*	ND	-
β-Glucosidase	35.0	33.4	1.05

ND*; not detected

In the ^{1}H NMR spectrum of it (Table III), the signals assignable to the aromatic protons were observed at δ 6.95-6.76, and those assignable to two methoxyl groups were also observed at δ 3.90 and 3.87. The signal at δ 4.88 assignable to anomeric proton of glucose were also observed. The large coupling constant (J = 7.3 Hz) of the anomeric proton in the glucoside suggests the β configuration for the anomeric center.

Table III. ^{1}H-NMR Spectral Data for 3,4-DMP Glucoside

H	δ	
2	6.92	(1H, d., J = 2.8 Hz)
5	6.95	(1H, d., J = 8.9 Hz)
6	6.76	(1H, q., J = 8.9, 2.8 Hz)
1'	4.88	(1H, d., J = 7.3 Hz)
2'-5'	3.38-3.70	(4H, m.)
6'a	4.02	(1H, dd., J = 12.0, 2.0 Hz)
6'b	3.98	(1H, dd., J = 12.0, 5.5 Hz)
3-OCH_3	3.90	(3H, s.)
4-OCH_3	3.87	(3H, s.)

Consequently, the glucoside can be identified as 3,4-dimethoxyphenyl-*O*-β-D-glucopyranoside (Fig. 4).

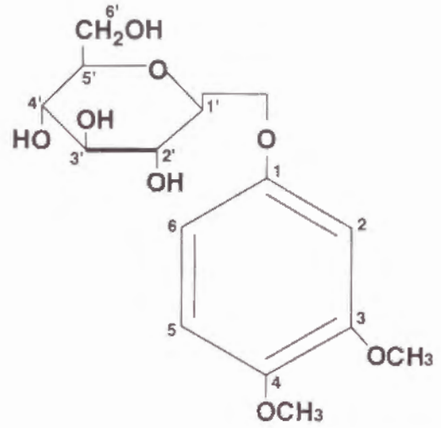


Fig. 4. Structure of 3,4-Dimethoxyphenyl-*O*-β-D-glucopyranoside.

Glucosylation of methoxyphenol derivative

In order to evaluate specificity of glucosylation of *C. arabica* cells, eight kinds of methoxyphenol derivatives including 3,4-DMP were tested as substrates.

Figure 5 shows typical HPLC chromatograms. In the case of 3,4-DMP, new peaks other than those of the substrate were detected. Since these peaks, which are marked with letter G in Fig. 5, were not detected in *C. arabica* cells without feeding substrate (Fig. 5-a), and were disappeared by hydrolysis with β-glucosidase (Fig. 5-b and 5-c), these peaks were considered to be the glucosylated products.

As for di and trimethoxyphenols, the efficiency of glucosylation at 48 h-culture was the greatest using 3,4-DMP as a substrate, and each value was 36% for 3,4-DMP, 35% for 3,5-DMP, 23% for 2,3-DMP, 13% for 2,6-DMP, and 26% for 3,4,5-TMP, respectively. In the case of 2,3-DMP, 2,6-DMP, and 3,4,5-TMP, two or three glucosides were detected. It suggested that *C. acrabca* cells can produce not only monoglucoside but also di and triglucosides. In addition, the value for monomethoxyphenols (*o*-MMP: 9%, *m*-MMP: 12%, *p*-MMP: 0%) were smaller than that for dimethoxyphenols. The cells could not glucosylate *p*-MMP during 2 weeks of culture.

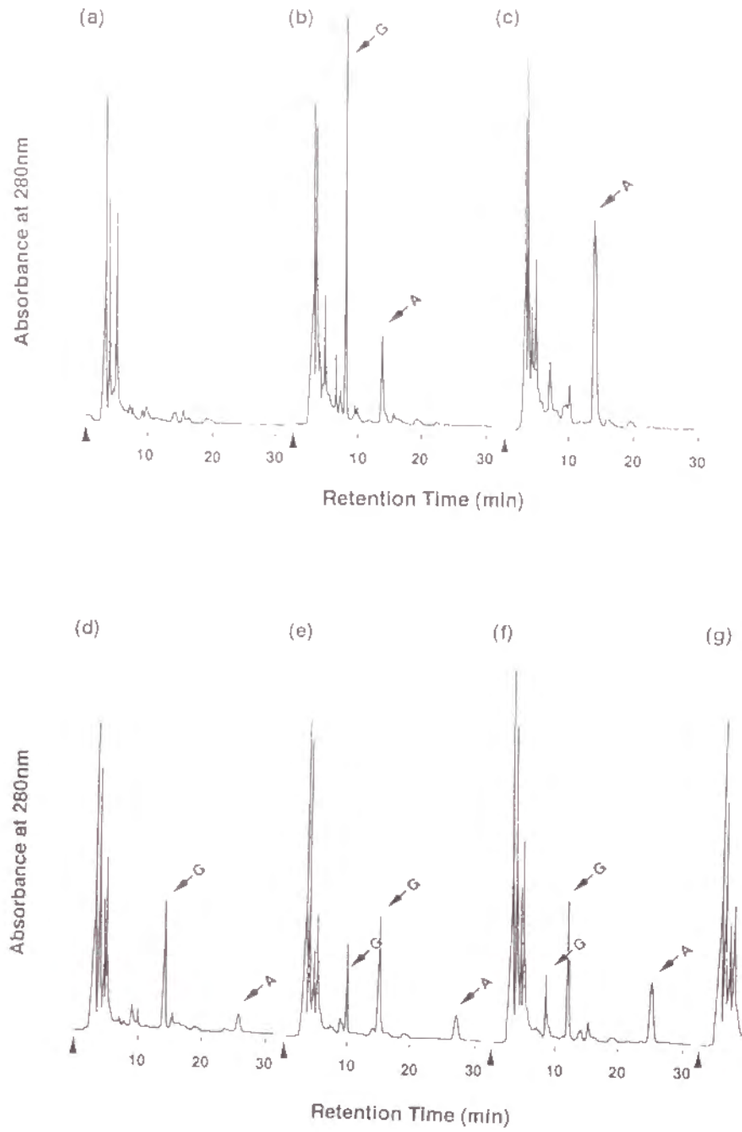


Fig. 5. HPLC Patterns in Glucosylation of Methoxyphenol Derivatives by *C. arabica* Cells.

C. arabica cells were cultured for 48 h, (a), without feeding substrate; (b), with 3,4-DMP; (c), with 3,4-DMP following β-glucosidase treatment; (d), with 3,5-DMP; (e), with 2,3-DMP; (f), with 2,6-DMP; (g), with 3,4,5-TMP. A, showed the peak of substrate; G, showed that of glucosides formed; ▲, showed the starting point of the chromatogram.

Discussion

Many plants contain useful glycosides such as pharmacologically active components of Chinese medicines. However, as they are existed very small quantities in the plants, it is very difficult to purify them in an industrial scale. To produce such useful glycosides, it is thought to be three methods. The first is the chemical synthesis, but it has a multiple-step reaction consisting acetylation, glycosylation, and deacetylation. And it always accompanied with some byproducts. The second is the enzymatic synthesis, but there are only a few reports which glycosylate phenolic compounds, and the resultant glycosides had α-linkage.[9,14] The third is glycosylation by cultured plant cells, which is a one-step and position-specific reaction. They made the glycosides with β-linkage, which was the same structure as those existing in nature. Therefore, glycosylation by cultured plant cells seems to be suitable for the purpose of this study. The author tried to produce 3,4-DMP glucoside, which had inhibitory activity of glucose absorption from the small intestines, by plant cell cultures from 3,4-DMP which easily and cheaply obtained.

In this section, five different plant cell cultures were tested for their abilities to glucosylate 3,4-DMP. Remarkable differences in glucosylation ability were found among these cultured cells. This result suggested the necessity of selecting for cells that are able to glucosylate an administered compound at a high rate. *C. arabica* cells were found to be suitable to this study, because they had a superior glucosylating ability and normally lack phenolic glucosides, such as 3,4-DMP glucoside. The present experiments showed that more than 40% of 3,4-DMP was converted into the corresponding

glucoside, when it was applied to *C. arabica* cells cultured on modified MS medium supplemented with 5 μM 2,4-D and 0.5 μM Kin. The accumulation of the glucoside in the cells was at its maximum after 96 h, and then gradually decreased until it was almost 20% by the end of growth cycle (2 weeks of culture). The glucoside seemed to be metabolized to other compounds. This view was supported by the evidence that neither release of glucoside from the cells into the medium nor reappearance of 3,4-DMP in the cultures was observed after that.

Seventy-nine mg of purified 3,4-DMP glucoside was obtained from 2000 ml-culture of *C. arabica* cells (200 ml packed cell volume), which yield was about 25%. On the other hand, it was reported that less than 5 g of partially purified 3,4-DMP glucoside was gained from 3000 g of crude black sugar (crude crystallized sugar from sugar cane juice),[78] which yield was about 0.2%. To gain 3,4-DMP glucoside, the method using cultured plant cells is more effective than that purified from sugar cane. Furthermore, the substrate, 3,4-DMP, can obtain in a large quantity and glucosylation of it can be done by cultured plant cells at a large scale. Therefore, it can be expected that 3,4-DMP glucoside might be used as an ingredient on a diet.

Eight methoxyphenol derivatives were used as a substrate for glucosylation by *C. arabica* cells. Especially, 3,4-DMP and 3,5-DMP were most effectively converted into their glucosides. The efficiency of glucosylation for dimethoxyphenols tended to be greater than that for monomethoxyphenols. After the addition of monomethoxyphenols, cells were begun to be necrosis within 24 h. On the other hand, cells added

dimethoxyphenols or trimethoxyphenol were not changed even in 96 h-culture. It suggested that monomethoxyphenols were more toxic for cells than dimethoxyphenols or trimethoxyphenol. This phenomenon seems to refer to the efficiency of glucosylation.

As regards dimethoxyphenols, the efficiency of glucosylation tended to decrease in the order of 3,4-DMP or 3,5-DMP, 2,3-DMP and 2,6-DMP. It suggested that the compounds not having methoxy group at ortho position were glucosylated more effectively than those having methoxy group at ortho position. The position of methoxy groups in the substrate molecules seems to be affective for glucosylation.

In this study, the author obtained seven kinds of glucoside including 3,4-DMP glucoside which reduced the level of plasma insulin, from eight kinds of methoxyphenol derivatives. Further study for this biological activity of these glucosides is needed.

Summary

3,4-Dimethoxyphenol (3,4-DMP) was converted into the corresponding glucoside in suspension-cultured cells of *Coffea arabica*. The maximum efficiency of glucosylation was attained more than 40% within 96 hours after the addition of 1 mM 3,4-DMP when cultured in a modified Murashige and Skoog's medium supplemented with 5 μM 2,4-dichlorophenoxyacetic acid and 0.5 μM kinetin. The glucoside was identified as 3,4-dimethoxyphenyl-*O*-β-D-glucopyranoside (3,4-DMP glucoside) by ^{1}H-NMR and hydrolysis with α- and β-glucosidases. In the study of substrate specificity of glucosylation using various methoxyphenol derivatives, seven kinds of glucosides including 3,4-DMP glucoside were obtained from eight kinds of the derivatives by *C. arabica* cells.

CONCLUSION

This thesis deals with biosynthesis of novel glycosides by microbial enzymes and cultured plant cells, and their utilization for food manufacturing. The results and findings obtained can be concluded as follows.

In Chapter I, the author described synthesis of glycosides by cyclodextrin glucanotransferase [1,4-α-D-glucan 4-α-D-(1,4-α-D-glucano)-transferase (cyclizing), EC 2.4.1.19, CGTase], and utilization of the glycosides formed.

A novel CGTase was purified from an alkalophilic *Bacillus* species. The purified enzyme had cyclizing activity, transglycosylating (coupling) activity, and starch-hydrolyzing activity, and these pH-activity curves had a single peak (pH 5.5 as the optimum pH) with a broad shoulder at alkaline pHs.

As the enzyme had such an alkali-tolerant property, when transglycosylation to various flavonoids was more effective at alkaline pHs than at neutral or acidic pHs, because of higher solubility of the flavonoids. Among flavonoids, hesperidin was transglycosylated by the CGTase, and hesperidin monoglucoside and a series of its oligoglucosides were produced. The structure of the purified monoglucoside was identified as 4^G-α-D-glucopyranosyl hesperidin by FAB-MS, α-, β-glucosidase, and glucoamylase treatments, and methylation analysis. And the solubilty of both purified hesperidin mono and diglucoside in water was remarkably improved.

In order to apply hesperidin glycosides to food manufacturing, effective method for their synthesis was developed using β-cyclodextrin (β-CD) and α-rhamnosidase. In transglycosylation reaction, β-CD was much effective for solubilization of hesperidin and synthesis of hesperidin glycosides by CGTase. In purification steps, as α-rhamnosidase hydrolyzed only unreacted hesperidin but did not hesperidin glycosides, hesperidin glycosides was easily separated from unreacted hesperidin.

By prepared hesperidin glycosides, various kinds of natural pigments were stabilized against ultraviolet radiation, and this result showed the possibility of application the glycosides to food manufacturing.

In addition, as CGTase from *Bacillus macerans* was found to transglycosylate various glycosides to make polyglucosylated products, which precipitated at 4 °C spontaneously, the method for a simple preparation of various glycosides from crude extracts was developed.

In Chapter II, the author described synthesis of glycosides by cultured plant cells and their properties.

Among many cultured plant cells, suspension-cultured cells of *C. arabica* were selected, because of their high glycosylation ability.

Vanillin was converted into the corresponding glucoside by *C. arabica* cells. The glucoside was identified as 4-formyl-2-methoxyphenyl-α-D-glucopyranoside by ^{1}H-NMR, ^{13}C-NMR, FAB-MS, and hydrolysis with α- and β-glucosidases. It retained the antimutagenic and antimicrobial activities of vanillin.

Capsaicin was also converted into the corresponding glucoside by *C. arabica* cells. The glucoside was identified as capsaicin-β-D-glucopyranoside by FAB-MS, ^{1}H-NMR, and hydrolysis with α- and β-glucosidases. The pungency of the glucoside was approximately 1/100 of that of capsaicin.

3,4-Dimethoxyphenol (3,4-DMP) was converted into the corresponding glucoside by *C. arabica* cells. By ^{1}H-NMR and hydrolysis with α- and β-glucosidases, the glucoside was identified as 3,4-dimethoxyphenyl-*O*-β-D-glucopyranoside (3,4-DMP glucoside), which had inhibitory activity of glucose absorption from the small intestines. From eight kinds of methoxyphenol derivatives, seven corresponding glucosides including 3,4-DMP glucoside could be obtained by glucosylation with *C. arabica* cells.

In conclusion, the author succeeded transglycosylation in alkaline pH range for the first time using a new CGTase from an alkalophilic *Bacillus* sp, and application of flavonoid glycosides formed to food manufacturing. Using CGTase from *B. macerans*, a new method for a simple preparation of various glycosides from crude extracts was developed. In addition, by suspension-cultured cells of *C. arabica*, various food components, which had phenolic OH groups, were glycosylated at a high rate.

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