

# Chapter 1 Reviews: Enzymatic Synthesis of -Glucosides Using Various Enzymes

## 1.1 Introduction

In recent years, transglycosylation or reverse hydrolysis reactions catalyzed by glycosidases have been applied to *in vitro* synthesis of oligosaccharides<sup>1-8)</sup> and alkylglycosides<sup>7-14)</sup>. Glucosylation is considered to be one of the important methods for the structural modification of compounds having -OH groups with useful biological activities since it increases water solubility and improves pharmacological properties of the original compounds. Enzymatic synthesis is superior to the chemical synthesis methods in such cases that the enzymatic reactions proceed regioselectively and stereoselectively without protection and deprotection processes. In addition, the enzymatic reactions occur usually under mild conditions: at ordinary temperature and pressure, and a pH value around neutrality. Various compounds, such as drugs<sup>13, 17)</sup>, vitamins and their analogues<sup>15, 16)</sup>, and phenolic compounds<sup>17)</sup>, have been anomer-selectively glucosylated by microbial glycosidases.

In this chapter, the methods for enzymatic synthesis of several glucosides and mechanism of xanthan gum synthesis by *Xanthomonas campestris* are reviewed in details.

## **1.2 Enzymatic synthesis of glucosides**

### **1.2.1 Neohesperidin -glucoside synthesis using cyclodextrin glucanotransferase of *Bacillus* sp. A2-5a<sup>18)</sup>**

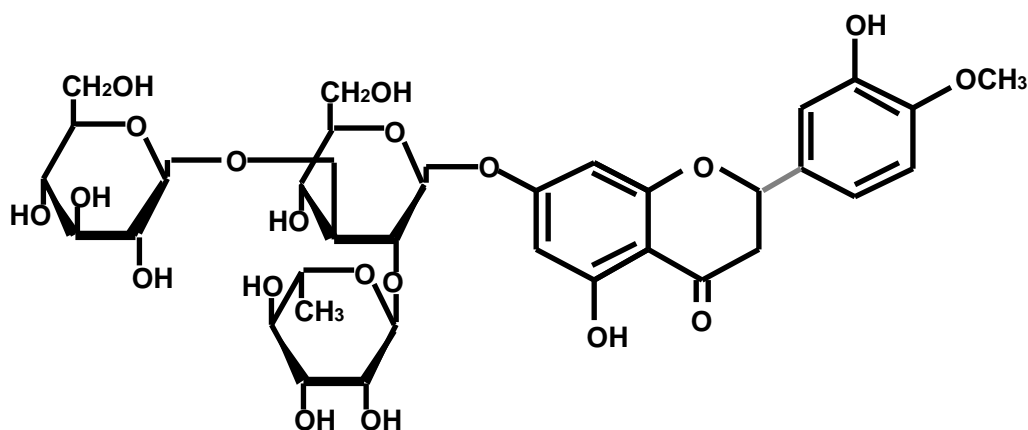
Citrus fruits contain two groups of flavonoid glycosides that have either rutinose (L-rhaminopyranosyl- -1, 6-glucopyranoside) or neohesperidose (L-rhaminopyranosyl- -1, 2-glucopyranoside) as their saccharide components. Hesperidin from mandarin oranges is tasteless. Neohesperidin from grapefruits is intensely bitter and important in citrus juices since it is converted into sweet dihydrochalcone derivatives by hydrogenation. However, since their solubilities in water are very low, enzymatic modification of neohesperidin was expected for applications in the food industry.

Kometani et al.<sup>18)</sup> carried out glucosylation of neohesperidin in an basic pH range using cyclodextrin glucanotransferase (1, 4- -D-Glucan 4- -D-(1, 4-glucano) transferase (cyclizing), EC 2.4.1.19; CGTase) from an alkalophilic *Bacillus* sp. A2-5a, because neohesperidin was more soluble at basic pHs than at neutral or acidic pHs.

Glucosylation of neohesperidin was carried out under the following standard reaction conditions. The reaction mixture (1 ml), containing 0.5% (w/v) neohesperidin as an acceptor for substrate, 5% (w/v) soluble starch as a donor, and 2 unit (soluble starch hydrolysis) of CGTase, was incubated at 40 and pH 10. After 16 h, the reaction mixture was treated with glucoamylase from *Rhizopus* sp. The glucosyl transfer product was purified by silica column

chromatography and HPLC, and the molecular structure of the product was identified as 3- $\beta$ -D-glucopyranosyl neohesperidin (neohesperidin monoglucoside, as shown in Fig. 1.1) by FAB-MS and NMR. The maximum molar conversion yield based on the amount of neohesperidin supplied reached 9.5%.

The solubility of neohesperidin monoglucoside in water ( $1.2 \times 10^2$  mg/ml) was approximately  $2.0 \times 10^3$  times higher than that of neohesperidin ( $6.1 \times 10^{-2}$  mg/ml). On the other hand, the bitterness of neohesperidin monoglucoside was approximately 10 times less than that of neohesperidin (data not shown). Such properties of neohesperidin monoglucoside are interesting and favorable for the use as a novel food additive.



**Fig. 1.1. Structure of neohesperidin monoglucoside.**

### 1.2.2 UDP-glucose synthesis using sucrose synthase of rice<sup>19)</sup>

Sucrose synthase (EC 2.4.1.13) is one of the most important enzymes of sucrose metabolism in plants, and is mainly responsible for the synthesis of nucleotide sugars by the cleavage of sucrose with nucleosidediphosphates<sup>20)</sup>. *In vivo*, UDP serves as substrate to yield UDP-glucose :

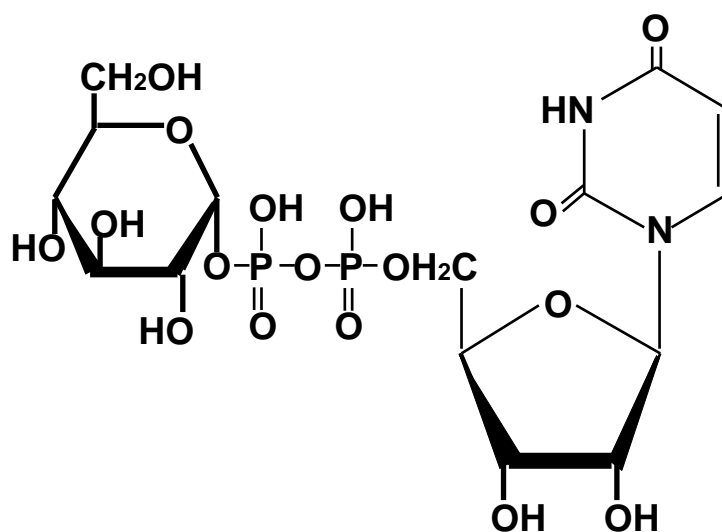


UDP-glucose is metabolized to secondary UDP sugars, such as UDP-glucuronic acid, UDP-xylose, and UDP-arabinose<sup>21)</sup>. UDP-glucose is also an important intermediate in sucrose-starch transformation in plants<sup>22)</sup>.

Sucrose synthase has been isolated and characterized from storage organs of different plants<sup>22)</sup>. Avigad<sup>22)</sup> described and reviewed a broad specificity of sucrose synthase of different plants for the cleavage of sucrose with different nucleosidediphosphates. The nucleosidediphosphate specificity of sucrose synthase has been investigated for UDP, ADP, and only in a few cases for TDP (for review, Ref. 22)). From the physiological role of sucrose synthase in plants, the enzyme seems to be applicable for enzymatic synthesis of UDP- and TDP-glucose. As for the enzymes used for synthesis of UDP-glucose, in comparison to the pyrophosphorylases<sup>23)</sup>, sucrose synthase has the advantage of no requirement for regeneration of the nucleosidetriphosphate.

With the objective to use sucrose synthase as a biocatalyst in the enzymatic synthesis of nucleotide sugars, Lothar et al.<sup>19)</sup> synthesized enzymatically UDP-glucose using sucrose synthase from rice. The conditions were optimized, and

UDP-glucose was synthesized as follows. Glucosylation of UDP was carried out under the following standard reaction conditions. The reaction mixture (1 ml) in 200 mM Hepes-NaOH buffer (pH 7.2), containing 1.57 mM UDP as an acceptor, 500 mM sucrose as a donor, and 1.8 mU of sucrose synthase, was incubated at 30 °C for 3 h. The reaction was terminated by heating at 95 °C for 5 min. The glucosyl transfer product was purified by HPLC, and the structure of the product was identified as uridine 5'-diphosphate (UDP-glucose, as shown in Fig. 1.2) by <sup>13</sup>C-NMR, <sup>1</sup>H-NMR and HMBC spectra. The maximum molar conversion yield based on the amount of UDP supplied reached 99%.



**Fig. 1.2. Structure of UDP-glucose.**

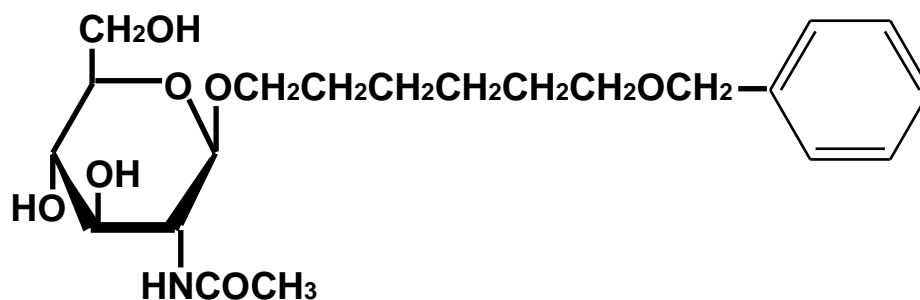
### 1.2.3 6-Benzoyloxyhexyl- $\beta$ -N-acetylglucosaminide synthesis using $\beta$ -N-acetylhexosaminidase of *Penicillium oxalicum*<sup>24)</sup>

$\beta$ -N-Acetylhexosaminidase (EC 3.2.1.52) is widely distributed in various mammalian tissues, higher plants, and microorganisms<sup>25)</sup>. The enzyme catalyzes hydrolysis of the  $\beta$ -N-acetylglucosaminyl or  $\beta$ -N-acetylgalactosaminyl moiety of the nonreducing end of oligosaccharides and of the sugar chains of glycoconjugates. Yamamoto et al.<sup>26)</sup> previously found a unique  $\beta$ -N-acetylhexosaminidase in the culture filtrate of *Penicillium oxalicum*, and succeeded in purifying the enzyme showing transglucosylation activity. The transglucosylation activity of  $\beta$ -N-acetylglucosaminidase (EC 3.2.1.30; generally shows low  $\beta$ -N-acetylgalactosaminidase activity) or  $\beta$ -N-acetylhexosaminidase has not been well-studied.  $\beta$ -N-Acetylhexosaminidase seems to be useful for the enzymatic synthesis of various oligosaccharides and complex carbohydrates including aminosugars since chemical synthesis of them is very intricate. Pochet et al.<sup>31)</sup> synthesized chemically 6-benzoyloxyhexyl-  $\beta$ -N-acetylglucosaminide (Fig. 1.3), which is useful as a drug carrier since it is an amphipathic compound (which can easily enter the cell through the lipid bilayer) consisting of a sugar moiety and alkyl chain, and used it as drug carrier for 3'-azido-3'-deoxythymidine (AZT), a potent anti-acquired immunodeficiency disease syndrome (AIDS) agent.

On the basis of the result by Pochet et al.<sup>31)</sup>, Kadowaki et al.<sup>24)</sup> synthesized enzymatically 6-benzoyloxyhexyl-  $\beta$ -N-acetylglucosaminide (Fig. 1.3) using  $\beta$ -N-acetylhexosaminidase of *P. oxalicum*, by the method described as follows.

Glucosylation of 6-benzyloxyhexane-1-ol was carried out under the following standard reaction conditions. The reaction mixture (0.05 ml), in 50 mM sodium citrate buffer (pH 4.5), containing 20% (w/v) 6-benzyloxyhexane-1-ol (organic synthesis) as an acceptor, 16 mM *N*-acetylchitotriose as a donor, and 0.08 Unit (*p*-nitrophenyl  $\beta$ -*N*-acetylglucosaminide hydrolysis) of  $\beta$ -*N*-acetylhexosaminidase, was incubated. The reaction was terminated by boiling in a water bath for 3 min. The glucosyl transfer product was purified by HPLC, and the structure of the product was identified as 6-benzyloxyhexyl-  $\beta$ -*N*-acetylglucosaminide (Fig. 1.3) by FAB-MS and <sup>1</sup>H-NMR.

Results reported by Pochet et al.<sup>31)</sup>, and Kadowaki et al.<sup>24)</sup> showed that the transglucosylation by  $\beta$ -*N*-acetylhexosaminidase might be useful for the synthesis of various biosurfactants such as alkylglucosaminide, for use as drug carriers, and various antibiotics having glycosides.



**Fig. 1.3. Structure of 6-benzyloxyhexyl-  $\beta$ -*N*-acetylglucosaminide.**

### 1.3 Xanthan gum synthesis by *Xanthomonas campestris*<sup>32)</sup>

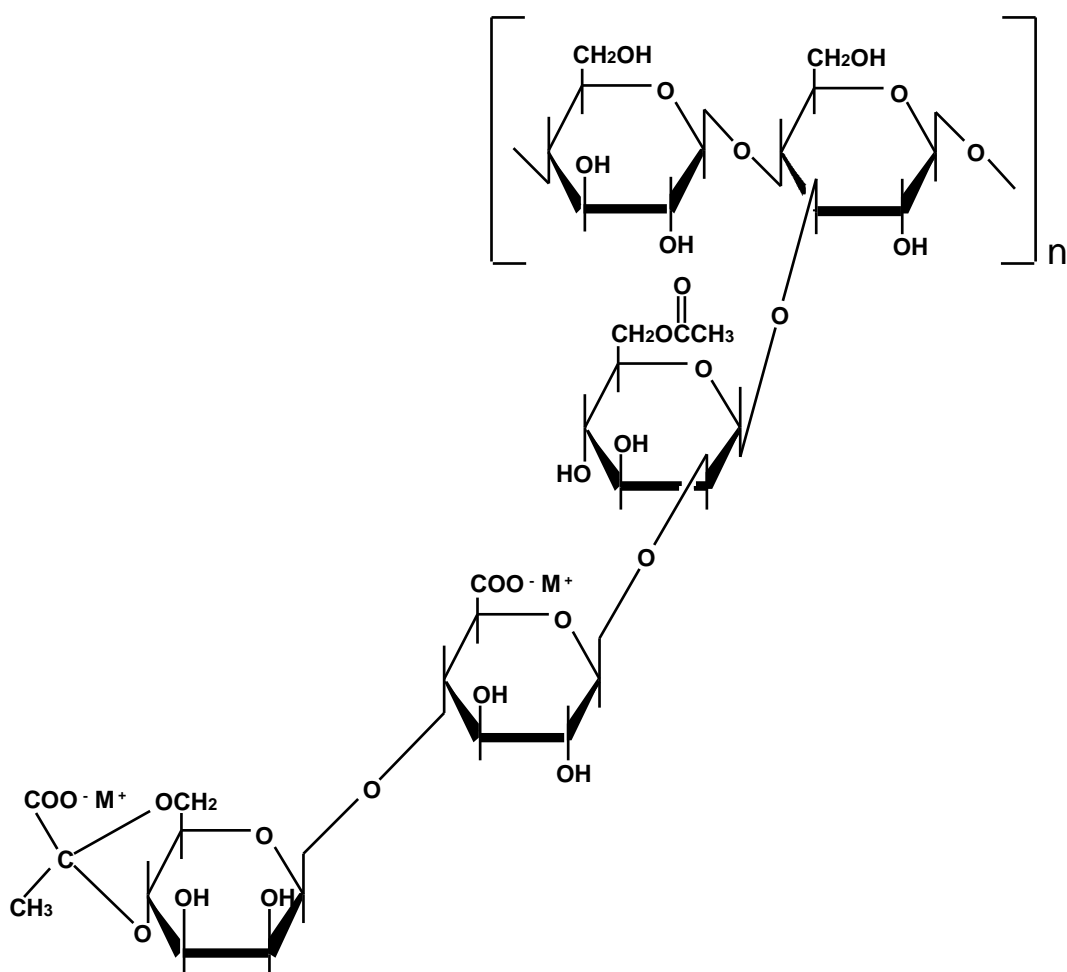
#### 1.3.1 Properties of xanthan gum

*Xanthomonas campestris* is a plant pathogenic bacterium that produces the exopolysaccharide xanthan gum. Xanthan gum has attracted particular attention because many applications have been found as a thickener and an emulsifier from its unique rheological properties in food industry as well as in oil recovery. Xanthan gum is an acidic polymer made up of pentasaccharide subunits, forming a cellulose backbone with trisaccharide side-chains composed of mannose (  $\rightarrow$  -1, 4) glucuronic-acid (  $\rightarrow$  -1, 2) mannose bonded to alternate glucose residues in the backbone by  $\rightarrow$  -1,3 linkages, as shown in Fig. 1.4<sup>33)</sup>. On approximately half of the terminal mannose residues, pyruvic acid moieties bond by a ketal linkage. Acetyl groups are often present as 6-*O* substituents on the internal mannose residues. Some external mannoses contain a second 6-*O*-acetyl substituent<sup>34)</sup>. The levels of pyruvate and acetyl substitution vary with bacterial growth conditions and other parameters.

#### 1.3.2 Biochemistry of xanthan synthesis

Xanthan gum is built up from cytoplasmic sugar nucleotides, acetyl-CoA, and phosphoenolpyruvate with an innermembrane polyisoprenol phosphate as an acceptor<sup>34-36)</sup>. The elucidation of its biosynthetic pathway has been greatly aided by using *X. campestris* cells frozen and thawed several





**Fig. 1.4. Structural unit of xanthan gum.**

times in the presence of EDTA. The stepwise assembly of the repeating pentasaccharide and the polymerization process were elucidated by the incubation of these permeabilized cells with combinations of UDP-glucose, GDP-mannose, and UDP-glucuronic acid. It was concluded that the synthesis of the repeating unit starts with the transfer of glucosyl 1-phosphate from UDP-glucose to polyisoprenol phosphate, followed by sequential transfer of the other

sugar residues to form the complete repeating unit. Acetyl and pyruvyl residues are bonded at the lipid-linked pentasaccharide level, donated by acetyl-CoA and phosphoenolpyruvate, respectively. The polymerization process indicated that xanthan chains grow at reducing end<sup>36)</sup>, as described for Wzy-dependent (formerly Rfc-dependent) *O*-antigen synthesis<sup>37)</sup>. The export mechanism of the polysaccharide remains to be determined but it might be associated to the polymerization process.

### **1.3.3 Genetics of xanthan synthesis.**

Many of the genetic methods used for research of *Escherichia coli* can be used in *X. campestris*, including conjugation, electroporation, chemical and transposon mutagenesis, and site-directed mutagenesis<sup>38-43)</sup>. The isolation of various non-mucoid mutants defective in xanthan production, generated by chemical mutagenesis or by transposon mutagenesis<sup>38-43)</sup>, revealed several genetic loci involved in xanthan biosynthesis.

Regions termed *xpsIII*, *xpsIV*, *xpsVI*<sup>43)</sup> as well as 35.3-kb gene cluster<sup>42)</sup> contain several genes required for the biosynthesis of sugar nucleotides necessary for both xanthan and lipopolysaccharide production. The 35.3-kb gene cluster comprises two separate regions, one of which overlaps the *xpsIII* region. The DNA sequence of a common segment of these regions revealed two genes, designated *xanA* and *xanB*<sup>44)</sup>. While the gene *xanA* encodes an enzyme with phosphoglucomutase and phosphomannomutase activities, *xanB* codes for a bifunctional enzyme with phosphomannose isomerase and GDP-mannose

pyrophosphorylase activities (Fig. 1.5). Thus, a portion of this region encodes two enzymes, one of which is required for the synthesis of all three sugar nucleotide precursors of xanthan, and the other for the synthesis of GDP-mannose. Genetic and biochemical analyses showed that mutations mapping in the *xpsIV* region affect the activity of the UDP-glucose pyrophosphorylase enzyme, whereas *xpsVI* mutations totally abolished UDP-glucose dehydrogenase levels (Fig. 1.5)<sup>43)</sup>. Genes encoding these enzymes have been recently sequenced<sup>45, 46)</sup>.

The genes required for lipid-linked intermediate assembly, polymerization, and secretion have been isolated and sequence<sup>39, 42)</sup>. They are clustered in a 16-kb region, termed *xpsI* or *gum* (Fig. 1.6). Unlike other exopolysaccharide synthetic systems, this region *gum* is unlinked from those required for the synthesis of sugar nucleotide precursors<sup>47-50)</sup>. Nucleotide sequence analysis predicted the presence of 12 open reading frames (*gumB* and *gumM*, as shown in Fig. 1.6)<sup>39, 51)</sup>. The transcriptional organization of *gum* region was analysed through *gum-lacZ* transcriptional fusions and primer-extension assays<sup>52)</sup>. These analyses indicate that the *gum* region is expressed as a single operon from a promoter located upstream of the first gene, *gumB*. A second promoter was identified upstream of *gumK*.

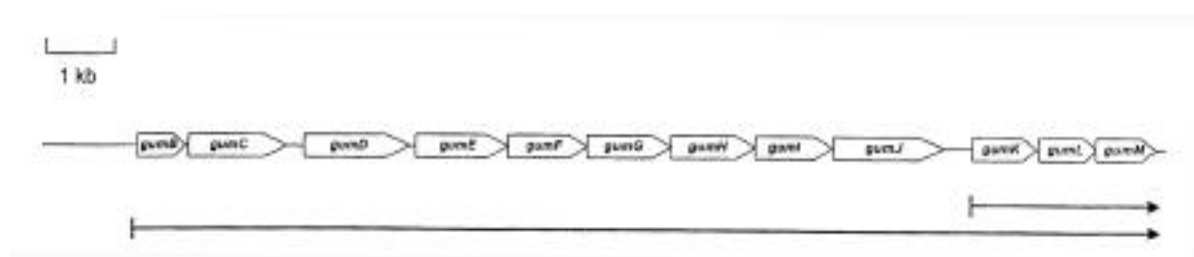
The biochemical functions of the *gum* gene products have been assigned by analyzing the *in vitro* formation of lipid-linked biosynthetic intermediates and polymers employing permeabilized cells *gum* mutant strains. The *gum* genes functions are summarized in Fig. 1.5. The GumD protein catalyzes the addition of glucose 1-phosphate to the polyisoprenol phosphate carrier. This reversible



reaction is the first step in the biosynthesis of Lipid-linked intermediates involved in the synthesis of xanthan. GumM catalyses the addition of  $\alpha$ -1, 4-glucose, followed by the internal  $\alpha$ -1, 3-mannose by GumH, a  $\alpha$ -1, 2-glucuronic acid by GumK, and the terminal  $\alpha$ -1, 4-mannose by GumI. The GumL protein incorporates pyruvyl residues to the external  $\alpha$ -mannose, while the acetyl residues are incorporated into the internal  $\alpha$ -mannose by GumF, and into the external  $\alpha$ -mannose by GumG.

In addition to the polyisoprenyl diphosphate pentasaccharide, the lipid-linked trisaccharide is able to act as a substrate for GumF. However, the lipid-linked acetyl trisaccharide can not act as an acceptor of a glucuronic acid residue, suggesting that the acetyl residues are incorporated into the polymer via the lipid-linked repeating unit.

Most of the *gum* genes could be disrupted within the wild-type strain. However, *gumB*, *gumC*, *gumE*, *gumM*, and *gumJ* genes could only be mutated by using a UDP-glucose-defective strain since their inactivation in a wild-type



**Fig. 1.6. Genetic map of the *X. campestris* *gum* operon showing the organization of the genes. Locations and designations of the genes are indicated as open boxes. Black arrows indicate the size and direction of the transcripts.**

background appeared to be lethal<sup>52, 53</sup>). Unexpectedly, the first step in the assembly of the lipid-linked intermediate was severely affected in these double mutants. This deficiency could be recovered by the introduction of a plasmid carrying the coding region for the C-terminal domain of GumD, which appeared to be responsible of its glucosyl-1-phosphate transferase activity<sup>53</sup>). These results suggest a possible regulatory role for GumD protein, or that a balanced expression of one or more proteins is required for the proper expression of the GumD activity. This may be of particular significance if GumD interacts with another protein. Since *gumB*, *gumC*, and *gumE* strains appear to accumulate complete xanthan subunits *in vitro* are unable to synthesize polymer, the products of these genes may be needed for polymerization or export the polymer. Although the function of the *gumJ* product can not be associated with a particular gum-biosynthetic step, a secretion role for GumJ can not be ruled out. Alternatively, it might be necessary for preventing accumulation of a harmful product or for recycling essential substrates.

#### **1.4 The objective of this thesis**

In this chapter, several studies as for glucoside synthesis using enzymes and mechanism of xanthan gum synthesis by *Xanthomonas campestris* have been described so far. The safety of *X. campestris* for use in food industry has been already well-known. In addition, the research group including the author successfully obtained *X. campestris* WU-9701 producing the -glucosyl

transfer enzyme. The enzyme was used for the  $\alpha$ -anomer-selective glucosylation of *l*-menthol with high yield of 99% using maltose as an  $\alpha$ -glucose donor. Moreover, it is interesting to note that the reaction by the enzyme of *X. campestris* WU-9701 produced no other  $\alpha$ -glucosides such as maltotriose and maltotetraose. These properties are different from those of general  $\alpha$ -glucosidases, which produced maltooligosaccharides in the reaction mixture and hydrolyzed maltose rapidly into glucose. These results suggested that the enzyme of *X. campestris* WU-9701 might be unique one different from usual  $\alpha$ -glucosidases producing maltooligosaccharide such as maltotriose and maltotetraose as  $\alpha$ -glucosyl transfer products from maltose.

In this thesis with the objective to characterize the  $\alpha$ -glucosyl transfer enzyme, the author purified it to give the single band on SDS-PAGE and determined enzymatic parameters. Moreover, the author cloned the gene (*xgtA*) encoding the  $\alpha$ -glucosyl transfer enzyme, catalyzing  $\alpha$ -anomer-selective glucosylation, and succeeded in the high-level expression of the gene *xgtA* in *Escherichia coli*. The recombinant enzyme XgtA produced by *E. coli* was utilized for efficient production of valuable  $\alpha$ -glucosides by  $\alpha$ -anomer-selective glucosylation reaction.

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## Chapter 2 Materials and methods

### 2.1 Introduction

In this chapter, some characteristics of *Xanthomonas campestris* WU-9701 used throughout the study, other microorganisms used, growth conditions for the microorganisms and methods of genetic manipulations, analyses and other experiments generally used for this thesis are described.

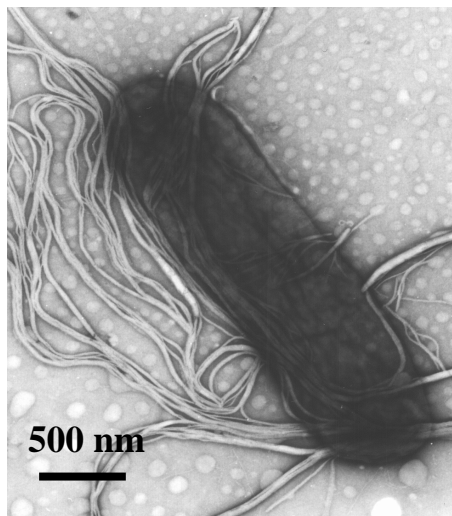
### 2.2 *X. campestris* WU-9701

*X. campestris* WU-9701, a producer of  $\alpha$ -glucosyl transfer enzyme, was used as a parental strain<sup>1)</sup> as shown in Fig. 2.1. The characteristics and morphology of the strain are shown in Table 2.1. *X. campestris* is employed as an industrial producer of xanthan gum, a natural and anionic heteropolysaccharide widely used as a suspension or thickening agent in food and pharmaceutical industries<sup>2,3)</sup>. Hence, the safety of *X. campestris* for use in food industry has already been well established.

### 2.3 *Escherichia coli*

As for the host of DNA manipulation and expression of *X. campestris*





**Fig. 2.1. Transmission Electron Micrograph of WU-9701.**

genes, *E. coli* JM109<sup>4)</sup> { *(lac-proAB) endA1 gyrA96 bsdR17 relA1 supE44 tbi*|F' *lacI<sup>q</sup> lacZ* M15 *proAB traD36*]} (Takara Shuzo Co. Ltd., Kyoto, Japan) was used.

## **2.4 Media, cultivation and strain maintenance of *X. campestris* WU-9701**

### **2.4.1 Cultivation of *X. campestris* WU-9701**

Cells of *X. campestris* WU-9701 were grown under the aerobic conditions with shaking at 30 °C for 48 h in one liter of medium as described in Table 2.2.

Table 2.1. Characteristics of *X. campestris* WU-9701.

Shape of cells	Rods
width ( $\mu$ m)	0.4-0.7
length ( $\mu$ m)	0.7-1.8
Motility	+
Number of flagella	1
Gram reaction	-
Lysis by 3% KOH	+
Aminopeptidase (Cerny)	+
Spores	-
Colony color	Yellow
Oxidase	-
Catalase	+
ONPG	N. T.
Alcohol dehydrogenase	N. T.
NO <sub>2</sub> from NO <sub>3</sub>	-
Urease	-
Hydrolysis of	
Gelatin	+
Tween 80	+
Esculin	+
Utilization of	
Glucose	+
Cellobiose	+
-Histidin	+
-Hydroxybutyrate	-

Symbols: ONPG, *o*-nitrophenyl -**D**-galactopyranoside;  
 + , positive; - , negative; N. T., not tested.

Using a sterile toothpick or loop, an individual colony of the cells from cell propagation on plate media was inoculated. The culture was incubated at 30 °C with shaking for 24 h. After 48 h cultivation, cells were harvested by centrifugation (15,000 × g, 30 min, 4 °C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0).

#### **2.4.2 Strain maintenance of *X. campestris* WU-9701**

Cryopreservation of 80% (v/v)-glycerol containing cell culture at -80 °C was used for preservation of *X. campestris* WU-9701. As short-term preservation, slants on 1.5% (g/v) agar medium as described in Table 2.2 were used, and subcultivation was done every month. The slants were stocked at 4 °C in a refrigerator.

### **2.5. Media, cultivation and strain maintenance of *Escherichia coli***

#### **2.5.1 Media for *E. coli***

For cultivation of *E. coli*, Luria-Bertani (LB) complete medium was used. The composition of LB medium is described in Table 2.3. For preparation of DNA competent-*E. coli*, M9 minimal medium was used for selection of F' strain. The composition of M9 medium is described in Table 2.4. When LB and M9 media were used as solid ones, agar 1.5% (w/v) was added and

Table 2.2. Medium for *X. campestris* WU-9701.

Content	(g / )
Maltose	50
Bacto-Peptide	10
Yeast extract	2
MgSO <sub>4</sub> • 7H <sub>2</sub> O	10
Initial pH	7.0

The pH was initially adjusted to 7.0 with 2.0 M NaOH.

sterilized. If necessary, antibiotics and vitamins at appropriate concentrations were added after cooling to room temperature.

### 2.5.2 Cultivation of *E. coli*

Cultivation of *E. coli* was done with LB medium, with addition of ampicillin at 25 mg/ml as an antibiotic, if necessary. Using a sterile toothpick or loop, an individual colony of the cells from cell propagation on plate media was inoculated. The culture was incubated at 37 °C with shaking for 16-18 h. The 3 ml aliquot of grown culture was transferred to 50 ml of LB media and cultivated at 37 °C with shaking for 1-3 h.

### 2.5.3 Strain maintenance of *E. coli*

Cryopreservation of 80% (v/v)-glycerol containing cell culture at -80 °C was

Table 2.3. LB (Luria-Bertani) medium (solution).

Content	(g / )
Bacto-Tryptone	10
Yeast extract	5
NaCl	10
Initial pH	7.0

The pH was initially adjusted to 7.0 with 2.0 M NaOH.

Table 2.4. M9 minimal medium (solution).

Content	
1M MgSO <sub>4</sub>	2 ml
20% (w/v) glucose	10 ml
0.1 M CaCl <sub>2</sub>	1 ml
1% (w/v) vitamin B1	1 ml
Na <sub>2</sub> HPO <sub>4</sub>	600 mg
KH <sub>2</sub> PO <sub>4</sub>	300 mg
NaCl	50 mg
NH <sub>4</sub> Cl	100 mg
Distilled water	to 1000 ml

also used for preservation of *E. coli*. As short-term preservation, LB-agar medium was used, and subcultivation was done every month. The host strains for transformation were spread once on M9-agar medium, and subcultivated to

LB-agar medium.

## **2.6 DNA extraction**

### **2.6.1 Total DNA extraction from *X. campestris* WU-9701**

To extract total DNA from *X. campestris* WU-9701, ISOPLANT (Nippon Gene, Toyama, Japan) was used. Finally, total DNA of *X. campestris* WU-9701 was precipitated by ethanol. After centrifugation at  $14,000 \times g$  for 30 min at 4 °C, resulting pellet of DNA was dissolved with 10 mM Tris-HCl - 1 mM EDTA (TE) buffer (pH 8.0).

### **2.6.2 Plasmid DNA extraction from *E. coli***

*E. coli* culture was poured into 1.5 ml microtubes and centrifuged at  $5,000 \times g$  for one min at 4 °C for removal of the medium completely. The cellular pellet was resuspended with 100 µl of TE buffer solution by vortexing. The chromosomal DNA was denatured by mixing the suspension completely with alkaline-SDS lysis solution. After neutralization of the mixture, plasmid DNA was extracted from water phase of the mixture by centrifugation and further purified. Purified DNA was dissolved and stored in TE buffer. For sequencing, GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, UK) was employed for purification of plasmid DNA.

### **2.6.3 Agarose gel electrophoresis**

To detect DNA band of total DNA of *X.campestris* WU-9701 or recombinant plasmids, agarose gel electrophoresis was performed. For the electrophoresis, 200 ml of 40 mM Tris-acetate-1 mM EDTA buffer (pH 8.0) was added to fill the electrophoresis tank and to cast agarose gel (1.0 % (w/v)). Then, 10 µl of DNA sample mixed with loading buffer was loaded into the slot of gel and electrophoresis was performed at 100 V for 35 min. After the electrophoresis, the agarose gel was dyed with ethidium bromide of 1 mg/ml for 10 min and the DNA band was detected by UV irradiation.

### **2.6.4 DNA recovery from agarose gel**

To recover specific electrophoreted DNA band or fractions from agarose gel, the aimed agar fractions were cut out with a sterile razor blade and taken into a microtube. Then, GFX PCR DNA and Gel Band Purification Kit capture buffer was added to the melted gel slices to trap DNA. Agarose gel was melted at 55 °C for 10 min, and finally the DNA was extracted in TE buffer.

## **2.7 Construction of a partial DNA library of *X. campestris* WU-9701**

To construct an *X. campestris* WU-9701 partial DNA library, a total DNA of *X. campestris* WU-9701 was digested with appropriate restriction enzymes,

isolated by agarose gel electrophoresis and recovered from agarose gel (2.5.3) to obtain DNA digests with appropriate fraction sizes. Then, the DNA digests with appropriate fraction sizes were purified by GFX PCR DNA and Gel Band Purification Kit (2.5.3). Finally, the DNA was dissolved again in TE buffer. For cloning of 4~6 kb DNA fractions, the purified DNA was ligated into the multicloning site of the plasmid vector pUC18 (Maxim Biotech, Inc., Canada).

## **2.8 Transformation of *E. coli***

The plasmid pUC18 ligated with 4~6 kb DNA fractions were used for transformation of *E. coli* JM109 by electroporation using 0.1 cm cell (Nippon Bio-Rad, Tokyo, Japan). The plasmid and *E. coli* JM109 were added into 0.1 cm cell, and the pulse was caused by a Gene Pulser (Nippon Bio-Rad, Tokyo, Japan) on this condition (1800 V, 400  $\mu$ s, 25  $\mu$ F). The recombinants grew as white colonies on LB agar supplemented with ampicillin, X-Gal and IPTG.

## **2.9 Gene cloning**

### **2.9.1 Oligonucleotide synthesis**

Oligonucleotides were synthesized by Invitrogen Co. Ltd. (Tokyo, Japan).



### 2.9.2 PCR (Polymerase chain reaction)

To obtain a gene (*xgtA*) encoding  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701, PCR was performed with a total DNA of *X. campestris* WU-9701 as a template. First, two oligonucleotide primers for use in the partial amplification of the *xgtA* by PCR were designed with reference to the N-terminal and internal amino acid sequences of the purified enzyme. The structures of degenerate primers specific for the 5'-converted region were 5'-CARACICCTGGTGGMG -3' and those specific for the internal region were 5'-AGIACYTGRTCKATCAT-3', where I, R, M, Y and K show deoxyinosine, A + G, A + C, C + T and G + T, respectively.

Total DNA (4  $\mu$ g) purified from *X. campestris* WU-9701 was used as a template in a 500  $\mu$ l of reaction mixture with 12.5 units of Taq polymerase (Nippon Roche, Tokyo, Japan). The amplification conditions were 95  $^{\circ}$ C for 3 min at the start, then 95  $^{\circ}$ C for 60 s, 49-56  $^{\circ}$ C for 60 s, and 72  $^{\circ}$ C for 150 s for total of 30 cycles. The 180-bp length PCR product, obtained as a single band on agarose gel electrophoresis, was used as a probe for screening the corresponding genes.

### 2.9.3 Colony hybridization

Colony hybridization was performed using a Hybond-N<sup>+</sup> membrane (Amersham, Buckinghamshire, UK) with a probe of approximately 180-bp fragment amplified by PCR (described above), and labeled with DIG-dUTP

(Boehringer Mannheim, Mannheim, Germany) as a probe under the stringent condition of 68 °C.

## **2.10 DNA sequencing**

The insert DNA of recombinant plasmid was sequenced by the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California, U.S.A) in accordance with the manufacture's instructions. An automatic ABI Prism 310 sequencer was used for electrophoresis. After obtaining the information about insert DNA sequence, new sequencing primers were designed from the sequence data until all of the insert sequence had been sequenced from both strands.

## **2.11 Southern hybridization**

### **2.11.1 Southern transfer**

DNA loaded on agarose-gel and subjected to electrophoresis was denatured by alkaline solution and then neutralized. Hybond-N membrane was placed on the gel and squashed down with thick blotting paper towel overnight to blot DNA in the gel onto the membrane. The membrane was air-dried, and the DNA was cross-linked to membrane by UV radiation<sup>5)</sup>.

### **2.11.2 Hybridization and detection**

The membrane blotted was prehybridized with the hybridization buffer for one hr, at 68 °C with gentle agitation. Then the membrane was incubated with fresh hybridization buffer of almost stringency, with digoxigenin (DIG) labeled probe (Boehringer Mannheim) added. Hybridization was performed at 68 °C for 8~16 h. Hybrid formation was detected by chemi-luminescence of alkaline phosphatase activity, which conjugated with anti-DIG antibody specifically cross-reacts to the DNA conjugated DIG.

## **2.12 Analytical methods**

### **2.12.1 Measurement of $\alpha$ -glucosides**

The amount of  $\alpha$ -glucoside in the filtrate was measured by High-Performance-Liquid Chromatography (HPLC) using the following conditions: column, TSK-Gel ODS 80-TS (4.6 × 250 mm, Tosoh Co., Tokyo); solvent, methanol-water (10:90, v/v); flow rate, 1.0 ml/min; and temperature, 40 °C. The amounts of glucose and maltose were measured by HPLC using the following conditions: column, Asahipak NH2P-50 4E (4.6 × 250 mm, Showa Denko. Co. Ltd., Tokyo); solvent, acetonitrile-10 mM tetra-*n*-propylammonium hydroxide containing acetic acid (pH 10.0) (70:30, v/v); flow rate, 1.0 ml/min; and temperature, 40 °C.

### 2.12.2 Spectrophotometric measurement

A spectrophotometric method was used for the determination of cell density, proteins and enzymes activities. Absorbance of the reaction mixture was measured by a double beam spectrophotometer (UV-210A, Shimadzu, Japan). The values of absorbance for optimum density of *E. coli* and *X. campestris* WU-9701 were 600 and 660, respectively.

### 2.12.3 Measurement of enzyme activities

-Glucosyl transfer activity was measured using hydroquinone as a substrate. Enzyme solution 100  $\mu$ l and 45 mM hydroquinone were added to 2 ml of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer containing 1.5 M maltose (pH 8.0) to give finally 1.2 M maltose in the reaction mixture, and incubated at 40 °C for 60 min. The reaction was stopped by heating at 100 °C for 10 min. The amount of hydroquinone  $\alpha$ -glucoside ( $\alpha$ -arbutin) formed was measured using HPLC. One unit (U) of  $\alpha$ -glucosyl transfer activity was defined as the amount of enzyme that produces one  $\mu$  mole of  $\alpha$ -arbutin per minute from hydroquinone under the conditions described above.

Maltose hydrolyzing activity was measured as the glucose-liberating activity using maltose as a substrate. Enzyme solution 100  $\mu$ l in 10 mM citrate-10 mM  $\text{Na}_2\text{HPO}_4$  buffer (pH 8.5) was added to 900  $\mu$ l of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer containing 1.1 M maltose (pH 8.5) to give finally 1.0 M maltose in the reaction mixture, and incubated at 40 °C for 60 min. The reaction was

stopped by heating at 100 °C for 10 min. Amount of glucose liberated was measured by HPLC. One unit (U) of maltose hydrolyzing activity was defined as the amount of enzyme that hydrolyzed one  $\mu$  mole of maltose per minute under the conditions described above.

#### **2.12.4 Measurement of protein concentration**

Protein content was determined by Coomassie Protein Assay Reagent (PIERCE Co. Ltd., Illinois, USA) with bovine serum albumin as a standard<sup>6)</sup>.

#### **2.12.5 Thin-layer-chromatography (TLC)**

Thin-layer chromatography (TLC) was performed using silica gel 60 plates (Merck Co., Darmstadt, Germany) with *n*-butanol-2-propanol-water (10:5:4, v/v/v) as the solvent. Spots were made visible by *p*-benzaldehyde-ethanol-H<sub>2</sub>SO<sub>4</sub> (1:19:1, v/v/v) followed by heating at 160 °C.

#### **2.12.6 NMR analysis**

<sup>13</sup>C-NMR, <sup>1</sup>H-NMR, <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) spectra were obtained using a JEOL JNM-LA 500 spectrometer (JEOL, Tokyo) operated at 150 MHz (<sup>13</sup>C-NMR) and 600 MHz (others) with sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) as an

internal standard.

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## Chapter 3     -Anomer-Selective Glucosylation of (+)-Catechin by the Crude Enzyme of *Xanthomonas campestris* WU-9701

### 3.1 Introduction

Catechins are polyphenol compounds composing the molecular structure of tannins, and widely distributed in nature, particularly in plants. Since they have many physiological functions such as antioxidative activity<sup>1)</sup>, antibacterial activity<sup>2)</sup>, and regulation of cholesterol levels in mammalian blood<sup>3)</sup>, many studies to identify the medicinal properties of catechins have been performed. However, the use of catechins is limited because they are poorly soluble in water and easily degraded by light irradiation in aqueous solution resulting in rapid browning<sup>4)</sup>.

Glycosylation is considered to be an important method for the structural modification of phenolic compounds with useful biological activities since it increases water solubility and improves pharmacological properties. Therefore, the glycosylated phenolic compounds have a possibility to be as prodrugs. In recent years, various phenolic compounds have been anomer-selectively glycosylated by microbial enzymes<sup>4-8)</sup>. Moreover, some enzymatic reactions generally proceed regioselectively and stereoselectively without protection and deprotection processes, and this facilitates anomer-selective glucosylation. In such cases, therefore, enzymatic synthesis is superior to organic synthesis



methods.

Recently, the author found that the lyophilized cells of *X. campestris* WU-9701 catalyzed the  $\alpha$ -anomer-selective glucosylation of  $\alpha$ -menthol using maltose as an  $\alpha$ -glucose supplier and efficiently produced  $\alpha$ -menthyl  $\beta$ -D-glucopyranoside ( $\alpha$ -MenG)<sup>9</sup>. The molar conversion yield based on the amount of  $\alpha$ -menthol supplied reached 99.1%. The author also found that *X. campestris* WU-9701 produces a unique enzyme showing  $\alpha$ -anomer-selective glucosyl transfer activity in the cells.

In this chapter, the author describe the selective production of (+)-catechin 3'-O- $\beta$ -D-glucopyranoside ( $\beta$ -C-G) through the reaction of the  $\alpha$ -anomer-selective glucosylation of (+)-catechin by the enzyme of *X. campestris* WU-9701. Since (+)-catechin has five -OH groups in its structure, as later shown in Fig. 3.3, (+)-catechin is a model substrate for determination of the regioselectivity of the WU-9701 enzyme. In addition, since (+)-catechin differs from  $\alpha$ -menthol which has only one -OH group in its structure and is insoluble in water. On the other hand, (+)-catechin has a bitter taste and this property, in some cases, makes it unsuitable for use in foods, particularly sweet foods. The author considered, therefore, that it may be useful if a (+)-catechin glucoside having no bitter taste synthesized using a simple method. For this objective, (+)-catechin  $\beta$ -glucosides might be desirable since  $\beta$ -glucosides generally have bitter taste.

## **3.2 Materials and Methods**

### **3.2.1 Materials**

(+)-Catechin was purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and maltose of special grade from Kanto Chemical Co., Inc. (Tokyo). All other chemicals used were commercially available and of a chemically pure grade.

### **3.2.2 Preparation of enzyme**

Cells of *X. campestris* WU-9701 were grown as described in Chap. 2. After 48 h of cultivation, cells were harvested by centrifugation ( $15,000 \times g$ , 30 min, 4 °C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). The wet cells (30 g) were suspended in 80 ml of the same buffer and the suspension was disrupted by sonication (20 kHz, 200 W, 120 min, 0 °C), and centrifuged ( $25,000 \times g$ , 60 min, 4 °C) again. The resulting supernatant (80 ml) was collected as the cell-free extract. The protein concentration of the cell-free extract was measured described in Chap. 2. The cell-free extract was appropriately diluted to contain 40 mg/ml protein and ammonium sulfate powder was added to 30% (w/v) saturation. The mixture was stirred at 4 °C overnight, and the precipitate was removed by centrifugation ( $25,000 \times g$ , 60 min, 4 °C). The supernatant was then adjusted to 50% (w/v) saturation with ammonium sulfate, and stirred at 4 °C overnight. The resulting precipitate was

collected by centrifugation ( $25,000 \times g$ , 60 min, 4 °C) and dissolved in 20 ml of 10 mM citrate-10 mM  $\text{Na}_2\text{HPO}_4$  buffer (pH 7.0). This solution was desalted through a Shephadex G-25 (Pharmacia, Buckinghamshire, UK) gel filtration column equilibrated with the same buffer. Fractions containing protein were detected based on the absorbance at 280 nm using a Shimadzu UV-240 spectrometer in Chap. 2. The fractions, with a total volume of 40 ml, showing glucose-transferring activity (corresponding to  $1.0 \times 10^4$  unit) from maltose were detected as described below and collected. These fractions were lyophilized, and 800 mg of crude enzyme was obtained.

### 3.2.3 Preparation of (+)-catechin glucoside

Unless otherwise indicated, glucosylation of (+)-catechin was carried out under the following standard reaction conditions. (+)-Catechin (60 mg) and the *X. campestris* crude enzyme (50 mg,  $6.5 \times 10^{-1}$  unit) were added to 10 ml of 10 mM citrate-10 mM  $\text{Na}_2\text{HPO}_4$  buffer (pH 6.5) containing 1.2 M maltose, and shaken at 180 oscillations per min at 45 °C for 24 h. Then, 0.5 ml of the reaction mixture was diluted with methanol up to 1.5 ml and filtered through a 0.2-  $\mu\text{m}$  PTFE membrane (Iwaki Glass Co., Ltd., Tokyo). The amounts of glucose, maltose, and (+)-C-G were measured by HPLC under conditions (A) and (B) described below.

### 3.2.4 Purification of catechin glucoside

The reaction mixture (10 ml) was extracted with 50 ml (10 ml each, five times) of ethylacetate to remove (+)-catechin. Following each extraction, the reaction mixture was centrifuged ( $2,000 \times g$ , 10 min, 4 °C) to clearly separate the organic and aqueous layers. (+)-Catechin was extracted into the organic layer, and (-)-C-G and saccharides remained in the aqueous layer. The aqueous layer was then extracted with 50 ml (10 ml each, five times) of *n*-butanol instead of ethylacetate in the same way as described above. The *n*-butanol layer containing (-)-C-G was collected and dried using a rotary evaporator. The resulting precipitate was redissolved in 5 ml of *n*-butanol, and loaded onto a silica column packed with Wakogel C-200 (Wako) with *n*-butanol-2-propanol-water (10:5:4, v/v/v) as the eluent. Fractions containing (-)-C-G were detected by TLC, collected, and dried using a rotary evaporator. The solid product, approximately 50 mg, was washed twice with 10 ml of *n*-hexane, and 48.1 mg of purified (-)-C-G was obtained. The molar conversion yield reached 51.4% based on the amount of (+)-catechin supplied.

### 3.2.5 Thin-layer chromatography (TLC)

TLC was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using the ascending method with *n*-butanol-2-propanol-water (10:5:4, v/v/v) as the solvent. Spots were made visible by spraying with *p*-methoxybenzaldehyde-ethanol-H<sub>2</sub>SO<sub>4</sub> (1:19:1, v/v/v) followed by heating at 160 °C.

### 3.2.6 High-performance liquid chromatography (HPLC)

HPLC was performed using a Shimadzu LC-6A system with an RID-6A differential refractometer (Shimadzu). To detect and measure catechin glucosides, the following conditions (A) were used: column, TSK-Gel ODS 80-TS (4.6 × 250 mm, Tosoh Co., Tokyo); solvent, methanol-water (20:80, v/v); flow rate, 1.0 ml/min; and temperature, 40 °C. To detect and measure saccharides such as glucose and maltose, the following conditions (B) were used: column, Asahipak NH2P-50 4E (4.6 × 250 mm, Showa Denko. Co., Ltd., Tokyo); solvent, acetonitrile-10 mM tetra-*n*-propylammonium hydroxide containing acetic acid (pH 10.0) (70:30, v/v); flow rate, 1.0 ml/min; and temperature, 30 °C.

### 3.2.7 NMR analysis

<sup>13</sup>C-NMR, <sup>1</sup>H-NMR and heteronuclear multiple bond coherence (HMBC) spectra were obtained using a JEOL JNM-LA 500 spectrometer (JEOL, Tokyo) operated at 125.65 MHz, 500 MHz, and 500 MHz, respectively, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

### 3.3 Results

#### 3.3.1 Preparation and isolation of (+)-3'-O- $\alpha$ -D-glucopyranoside

A typical TLC chromatogram of the reaction product is shown in Fig. 3.1. Since only one product with an *R<sub>f</sub>* value of 0.8 was presumed to be a (+)-catechin glucoside, it was extracted from the reaction mixture, purified by silica gel column chromatography, and subjected to NMR analysis.

Although the  $^{13}\text{C}$ -NMR and  $^1\text{H}$ -NMR spectra of the isolated product were obtained, significant changes in chemical shifts were not observed, except for the H2' and H6' signals, in comparison with those observed for (+)-catechin and  $\alpha$ -D-glucose (data not shown), similar to the finding that has also been described by other researchers<sup>10, 11</sup>). However,  $^{13}\text{C}$ -NMR and  $^1\text{H}$ -NMR spectral analyses alone were presumed insufficient for structure determination of the product, particularly to distinguish (+)-catechin 3'-O- $\alpha$ -D-glucopyranoside from (+)-catechin 4'-O- $\alpha$ -D-glucopyranoside as product candidates. Therefore, a two-dimensional HMBC spectrum was obtained. As shown in Fig. 3.2, a sequence of correlation at the C3'/H1" position was clearly detected, indicating that  $\alpha$ -D-glucose was bonded to the C3' position of (+)-catechin. Consequently, the isolated product was identified as (+)-catechin 3'-O- $\alpha$ -D-glucopyranoside ((+)-3'-O- $\alpha$ -D-glucopyranoside), and its structure is shown in Fig. 3.3.

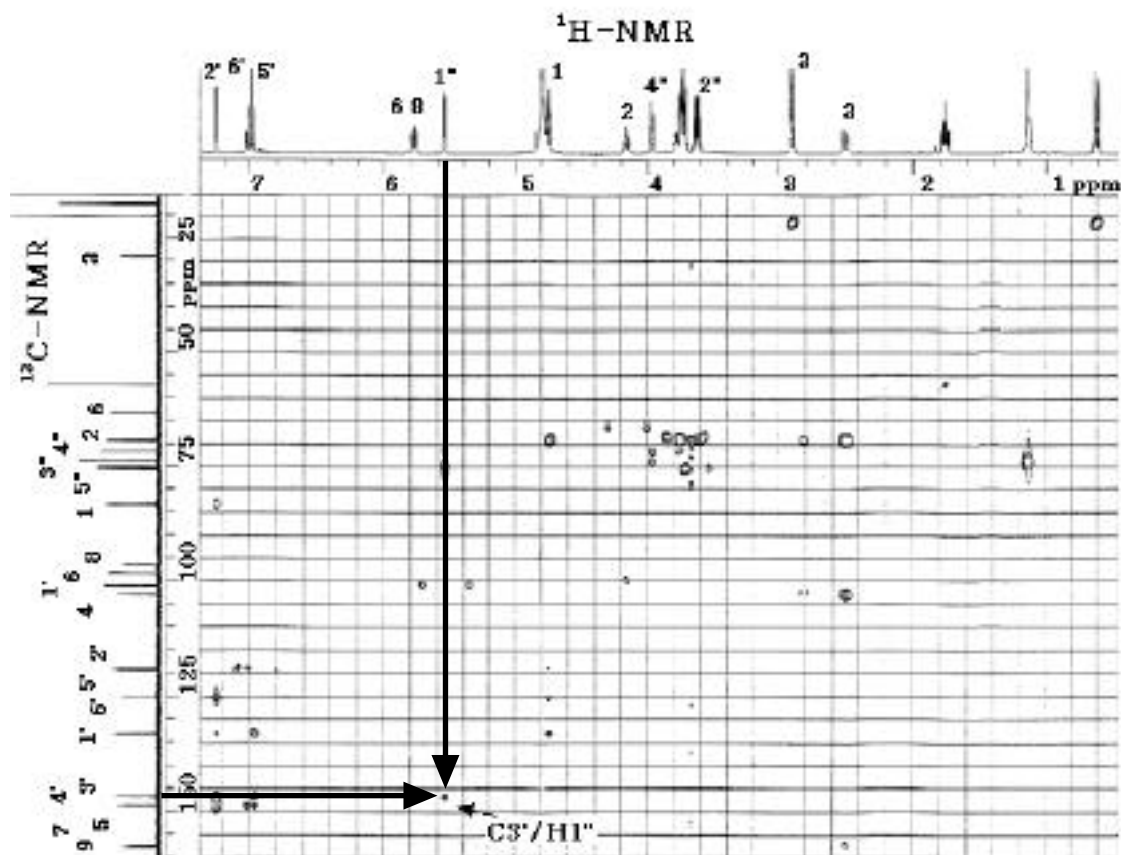
HPLC chromatograms of the reaction mixture are shown in Fig. 3.4. In the analysis using an ODS column (Fig. 3.4A), a new peak corresponding to (+)-3'-O- $\alpha$ -D-glucopyranoside at a retention time of 11.7 min was detected. In the analysis using an NH2P-50



**Fig. 3.1.** TLC of the reaction products from (+)-catechin and maltose using the crude enzyme of *X. campestris* WU-9701. The reaction conditions are described in Materials and Methods.

**Abbreviations:** C, (+)-catechin; P, product ( -C-G); G, glucose; S, saccharides. **Lanes:** 1, reaction mixture; 2, the reaction product purified after silica column chromatography. The *R<sub>f</sub>* values of (+)-catechin, the product ( -C-G), and glucose are 0.9, 0.8, and 0.3, respectively.

4E column, glucose and maltose were detected at the retention times of 5.8 and 7.7 min, respectively (Fig. 3.4B). Other oligosaccharides, such as maltotriose, or other glucosylated derivatives, such as (+)-catechin oligoglucosides (for example, -C-G-G), were not detected, similar to the situation for -MenG synthesis<sup>9</sup>).

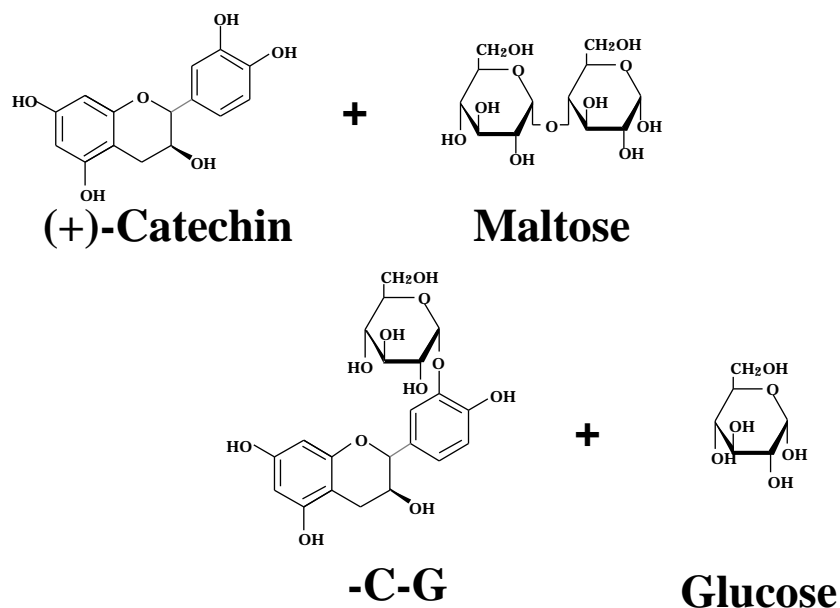


**Fig. 3.2.** Two-dimensional HMBC (heteronuclear multiple bond coherence) spectrum of the isolated product.  $^{13}\text{C}$ -NMR,  $^1\text{H}$ -NMR and HMBC spectra were obtained using a JEOL JNM-EX 500 NMR spectrometer operating at 125.65 Mz, 500 MHz and 500 MHz, respectively. Chemical shifts were expressed in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

### 3.3.2 Optimization of -C-G synthesis

The optimum reaction conditions were determined by sequentially changing the maltose concentration, pH, temperature and amount of (+)-catechin supplied.

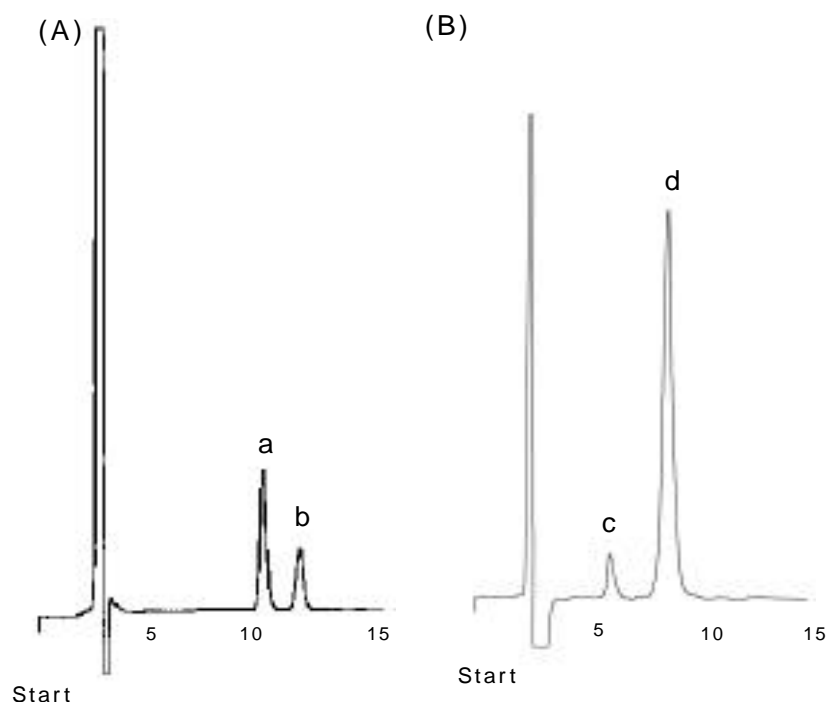




**Fig. 3.3. Structure of (+)-catechin 3'-O- -D-glucopyranoside ( -C-G) produced by the crude enzyme of *X. campestris* WU-9701.**

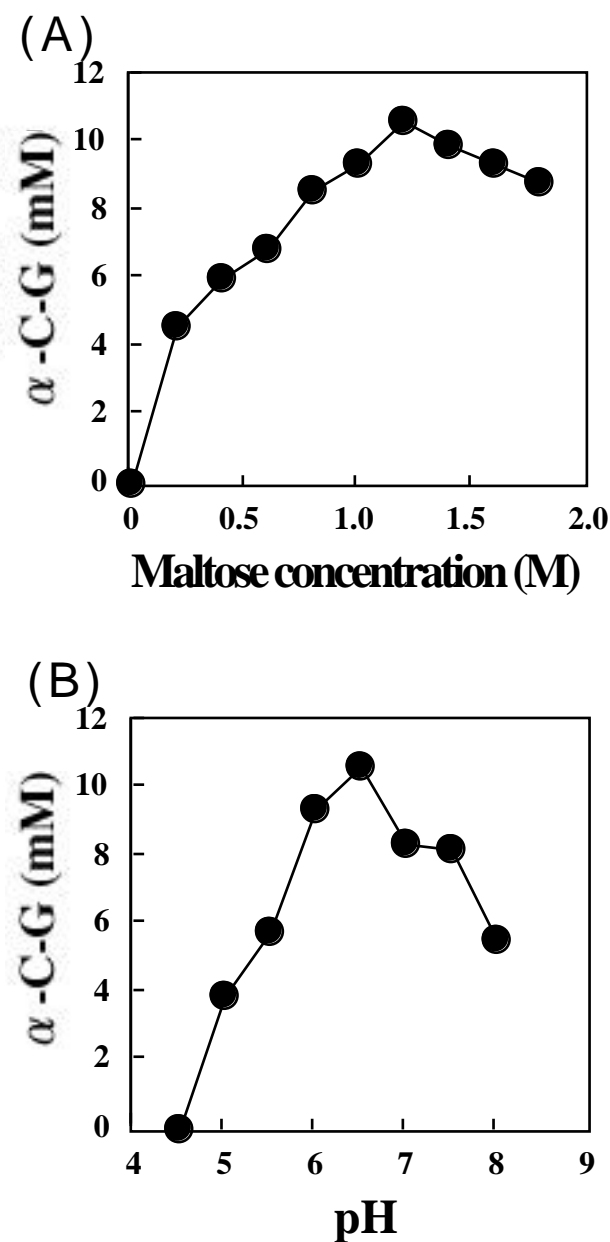
Fig. 3.5A shows the effects of maltose concentration on the synthesis of -C-G, and at 1.2 M a maximum of 48.1 mg of -C-G was formed, which corresponded to a molar conversion of 57.1% based on the amount of (+)-catechin supplied (60.0 mg). At the concentrations of maltose above 1.4 M, the production of -C-G gradually decreased.

As shown in Fig. 3.5B, the effects of pH on the synthesis of -C-G were determined. The amount of -C-G produced was highest at pH 6.5. Below pH 5.5 and over pH 7.0, production of -C-G decreased, probably due to the lower enzyme activity under these pH conditions. As shown in Fig. 3.5C, the effects of temperature on the synthesis of -C-G were determined. The amount of -C-G produced was highest at 45 °C. Since (+)-catechin was more soluble in hot

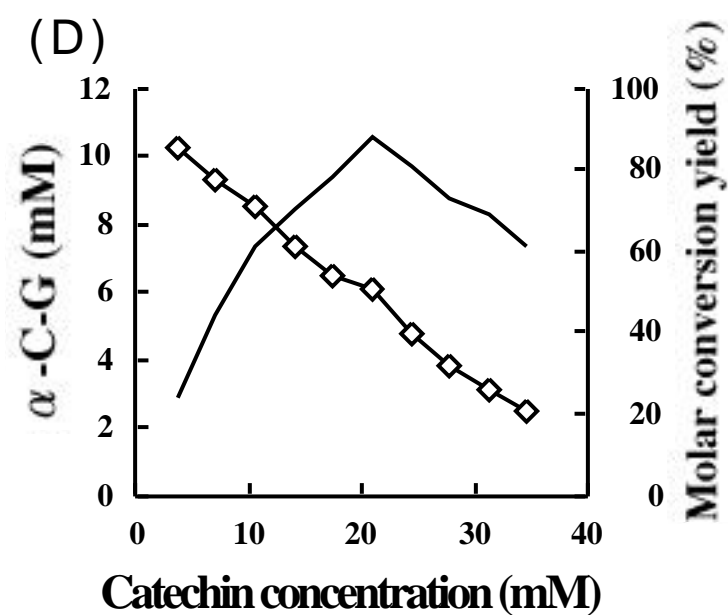
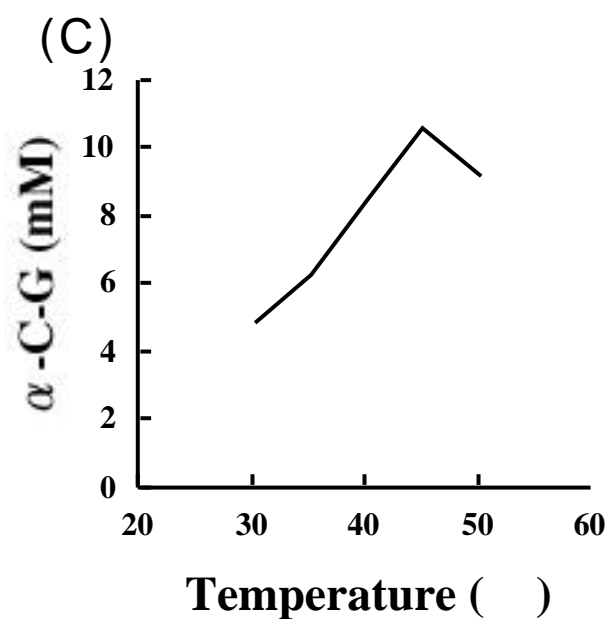


**Fig. 3.4. HPLC of the reaction products from (+)-catechin and maltose using the crude enzyme of *X. campestris* WU-9701. The reaction mixture was loaded onto (A) a TSK-Gel ODS-80TS column and (B) an Asahipak NH2P-50 4E column, details having been described in Materials and Methods. Peaks: a, (+)-catechin; b, -C-G; c, glucose; and d, maltose.**

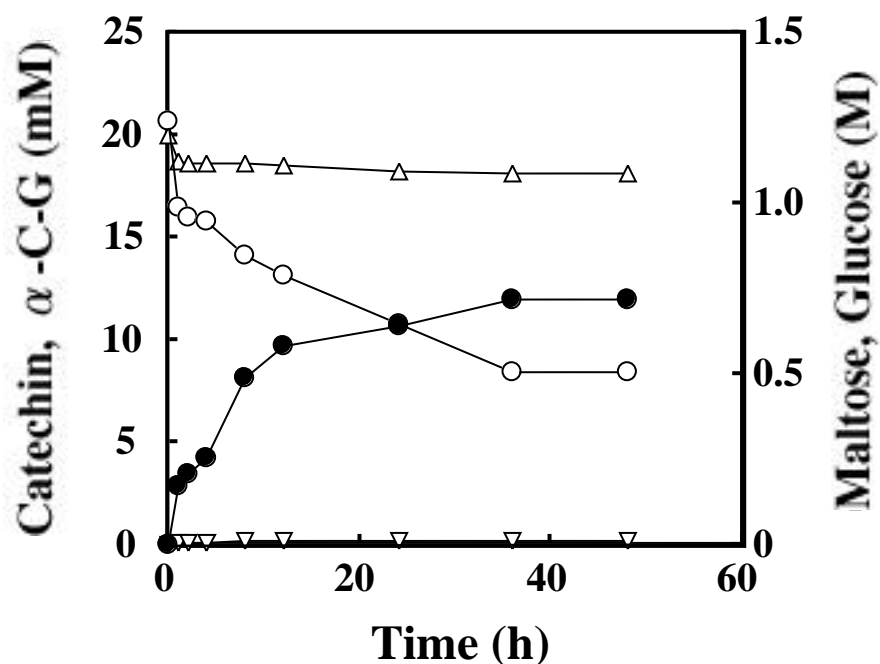
water than in cold water, a high temperature was thought to be more suitable for the synthesis of -C-G. However, over 50 °C, production of -C-G decreased, probably due to inactivation of the enzyme caused by heat denaturation. As shown in Fig. 3.5D, the effects of the amount of (+)-catechin supplied on the synthesis of -C-G were determined. When 5 mg of (+)-catechin was used, the highest molar conversion yield, 80%, was achieved. To obtain the highest possible production level of -C-G, the author changed the amount of (+)-catechin supplied, and the production of -C-G increased proportionately, reaching the highest level at 20 mM (60 mg/10 ml). Under these conditions,



**Fig. 3.5. Effects of maltose concentration (A), pH (B), temperature (C) and (+)-catechin concentration (D) on  $\alpha$ -C-G synthesis by the crude enzyme of *X. campestris* WU-9701. (A) Concentrations of maltose were changed in the standard reaction as described in Materials and Methods. (B) The pH of 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer was changed to obtain the standard reaction conditions as described in Materials and Methods. Symbols:  $\bullet$ ,  $\alpha$ -C-G.**



**Fig. 3.5 continued. (C) Temperature was changed in the standard reaction conditions as described in Materials and Methods. (D) (+)-Catechin concentration was changed in the standard reaction conditions as described in Materials and Methods. Symbols:  $\diamond$ ,  $\alpha$ -C-G; —, molar conversion yield.**



**Fig. 3.6.** Time course of  $\alpha$ -C-G synthesis by the crude enzyme of *X. campestris* WU-9701. The reactions were carried out under the standard reaction conditions: maltose, 1.2 M; pH, 6.5; temperature, 45 °C and (+)-catechin, 20 mM (6 mg/ml). Symbols:  $\circ$ ,  $\alpha$ -C-G;  $\bullet$ , (+)-catechin;  $\triangle$ , maltose;  $\nabla$ , glucose.

$\alpha$ -C-G production reached a maximum of 10.6 mM with a molar conversion yield of 51.4%.

Thus, the optimum conditions for the synthesis of  $\alpha$ -C-G were determined as the following: maltose concentration, 1.2 M; pH, 6.5; temperature, 45 °C; and amount of (+)-catechin supplied, 20 mM (60 mg/10 ml).

Fig. 3.6 shows the time course for the synthesis of  $\alpha$ -C-G under the optimum conditions. Production of  $\alpha$ -C-G increased as the reaction proceeded with a gradual decrease in maltose concentration, and the total amount of  $\alpha$ -C-G reached a maximum of 54.1 mg at 36 h, with a molar conversion yield of

57.1% based on the amount of (+)-catechin supplied. Under these conditions, only -C-G was synthesized and other glucosylated (+)-catechin derivatives, such as -C-G-G, were not detected, similar to the results shown in Fig. 3.4. Moreover, except for maltose, no other oligosaccharides, such as maltotriose or maltotetraose, were detected. At 36 h, -C-G formation seemed to stop, and thereafter the amount of -C-G remained constant and -C-G was not hydrolyzed.

### 3.3.3 Properties of -C-G

Since some properties of -C-G have been described by Kitao *et al.* (4), the author list additional and advantageous properties of -C-G in this paper. At 20 the solubility in pure water of -C-G, of 450 mg/ml, was approximately 100 fold higher than that of (+)-catechin, of 4.6 mg/ml. When 5 mM -C-G and 5 mM (+)-catechin were dissolved in 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0), both of the solutions were clear at time zero. However, as shown in Fig. 3.7A, after standing for 24 h in contact with air at 20 the solutions containing (+)-catechin turned brown. However, the solution containing -C-G showed no color change under the same conditions, as shown in Fig. 3.7B. These results indicate that -C-G is stable with respect to oxidation. Moreover, -C-G had no bitter taste and a slight sweet taste which differs from (+)-catechin which has a very bitter taste and no sweet taste. This property of -C-G may make it a desirable food additive, particularly sweet foods.



**Fig. 3.7. Stability of (+)-catechin and -C-G in solution. (+)-Catechin and -C-G were dissolved in 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). After 24 h in contact with air at 20 °C, the solution containing (+)-catechin (A) turned brown, whereas the one containing -C-G (B) did not.**

### 3.4 Discussion

In this study, (+)-catechin was efficiently and  $\alpha$ -anomer-selectively glucosylated by the crude enzyme, showing glucosyl transfer activity, of *X. campestris* WU-9701. A maximum molar conversion yield of 57.1% was achieved following a 36 h reaction under the optimum conditions.

In general, the enzymatic synthesis of glucosides is carried out via a transglucosylation reaction or the reverse hydrolysis reaction of glucosidases. Since the *X. campestris* enzyme could not form -C-G when glucose instead of maltose was used as a carbohydrate substrate (data not shown), it seems likely that -glucosylation of (+)-catechin occurred via a transglucosylation reaction

with maltose as an  $\alpha$ -glucose donor. In the transglucosylation reaction, a high concentration of the glucosyl donor is favorable, and in this study the optimum maltose concentration for  $\alpha$ -C-G production was 1.2 M as shown in Fig. 3.5A. However,  $\alpha$ -C-G synthesis by the crude enzyme of *X. campestris* WU-9701 occurred even at a concentration of maltose as low as 0.2 M (Fig. 3.5A). The high transglucosylation activity of this enzyme may be useful not only for the efficient production of  $\alpha$ -C-G, but also for the synthesis of commercially important  $\alpha$ -glucosides. Since other glucosylated products such as maltotriose or  $\alpha$ -C-G-G were not observed as shown in Figs. 3.4 and 3.6, there is the possibility that the enzyme prefers compounds having phenolic -OH groups, but not disaccharides, as acceptors.

In a previous study, the optimum pH for the synthesis of  $\alpha$ -MenG by lyophilized cells of *X. campestris* WU-9701 was 8.0<sup>9)</sup>. However, in this study,  $\alpha$ -C-G synthesis using the *X. campestris* enzyme was highest at pH 6.5. As described in the Introduction, (+)-catechin is unstable in water, particularly under alkaline conditions. Therefore, the optimum pHs are different for the syntheses of  $\alpha$ -MenG and  $\alpha$ -C-G, and the amount of  $\alpha$ -C-G produced decreased when the pH was above 7.0 (Fig. 3.5B).

To date, several researchers have reported on the enzymatic synthesis of  $\alpha$ -C-G using the purified enzyme<sup>4,6)</sup>. However, the use of the purified enzyme is generally expensive and seems difficult for bulky products on an industrial scale. On the other hand, the crude enzyme of *X. campestris* WU-9701 was prepared using a simple method as described in Materials and Methods, and it should be easy and cheap to use this method for the large-scale production of



-C-G. *X. campestris* is a typical strain used for the production of xanthan gum<sup>12)</sup>, and the safety of *X. campestris* for use in the food industry is well known. Since the reaction mixture used in the present study contains no components harmful to the human body, the reaction system using the *X. campestris* WU-9701 crude enzyme described in this study seems to be applicable for the production of -C-G as a food additive on an industrial scale. Moreover, this system might be useful for the -anomer-selective glucosylation of other polyphenols or phenolic compounds.

In conclusion, the author succeeded in establishing a simple and efficient method for the a-anomer-selective synthesis of -C-G. To our knowledge, the molar conversion yield of 57.1%, based on the amount of (+)-catechin supplied, is the highest reported to date<sup>4, 6, 8)</sup>. It is also interesting to note that the enzyme regio-selectively glucosylated -OH group at the C'3 position, but not the other -OH groups, of (+)-catechin. Thus, it seems that this enzyme has the ability to distinctively glucosylate -OH groups in acceptors having several -OH groups. Such unique properties of the *X. campestris* WU-9701 enzyme enabled us to selectively produce -C-G with a high yield.

### 3.5 References

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## Chapter 4 Enzymatic Synthesis of $\alpha$ -Arbutin by $\alpha$ -Anomer-Selective Glucosylation of Hydroquinone Using Lyophilized Cells of *Xanthomonas campestris* WU-9701

### 4.1 Introduction

Hydroquinone-*O*- $\alpha$ -D-glucopyranoside, commonly called as  $\alpha$ -arbutin, occurs in plants such as *Uvae ursi*, and is used in cosmetics since it has a whitening effect on the skin. On the other hand,  $\beta$ -arbutin, *i.e.*, hydroquinone-*O*- $\beta$ -D-glucopyranoside, is not a natural product. However,  $\beta$ -arbutin also has a whitening effect and the same level of inhibiting activity toward tyrosinase as  $\alpha$ -arbutin<sup>1,2)</sup>. Hence,  $\beta$ -arbutin was enzymatically synthesized from hydroquinone by several researchers using amylase of *Bacillus subtilis*<sup>3)</sup> and sucrose phospholylase of *Leuconostoc mesenteroides*<sup>4)</sup>.

With the objective to produce a useful derivative of  $\alpha$ -menthol, for the first time. Nakagawa et al. has succeeded in obtaining  $\alpha$ -menthyl  $\alpha$ -D-glucopyranoside ( $\alpha$ -MenG), an  $\alpha$ -glucosyl derivative of  $\alpha$ -menthol, through a one-step enzymatic synthesis using *Saccharomyces cerevisiae*  $\alpha$ -glucosidase<sup>5-7)</sup>. Moreover, in a previous study<sup>8)</sup>, Nakagawa et al. showed that lyophilized cells of *Xanthomonas campestris* WU-9701 catalyzed the  $\alpha$ -anomer-selective glucosylation of  $\alpha$ -menthol using maltose as an  $\alpha$ -glucose supplier and that they obtained  $\alpha$ -MenG as the only glucosylated product through a crystal accumulation reaction. The molar conversion yield based on the amount of  $\alpha$ -

menthol supplied reached 99%<sup>8)</sup>. On the other hand, as described in chapter 3, the author also used successfully the crude enzyme of *X. campestris* WU-9701 for  $\alpha$ -anomer-selective glucosylation of (+)-catechin : (+)-catechin 3'-O- $\beta$ -D-glucopyranoside was selectively produced at a molar conversion yield of 57%, based on the amount of (+)-catechin supplied. These results suggest that the enzyme of *X. campestris* WU-9701 is a unique biocatalyst applicable to the  $\alpha$ -anomer-selective glucosylation of organic compounds having alcoholic -OH groups.

Hydroquinone has two phenolic -OH groups at the *para* position in its structure and is an interesting model-compound for examination of enzymatic reactivity. In this chapter, the author describes the  $\alpha$ -anomer-selective glucosylation of hydroquinone, that is, the selective production of  $\alpha$ -arbutin from hydroquinone and maltose, by lyophilized cells of *X. campestris* WU-9701 according to the reaction scheme as described after in Fig. 4.3.

## 4.2 Materials and Methods

### 4.2.1 Materials

Hydroquinone and maltose were purchased from Kanto Chemical Co., Inc. (Tokyo). All other chemicals used were commercially available and of a chemically pure grade.

#### **4.2.2 Preparation of lyophilized cells of *X. campestris* WU-9701**

Cells of *X. campestris* WU-9701 were grown as described in Chap. 2 . After 48 h of cultivation, cells were harvested by centrifugation (15,000 × g, 30 min, 4 °C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). The wet cells were suspended in 40 ml of the same buffer and was lyophilized. The lyophilized cells showing 6.6 unit/mg-protein were stored at 4 °C in the refrigerator.

#### **4.2.3 Preparation of $\alpha$ -arbutin**

Unless otherwise indicated,  $\alpha$ -anomer selective glucosylation of hydroquinone was carried out under the following standard reaction conditions. Hydroquinone (45 mM) and lyophilized cells of *X. campestris* WU-9701 ( $6.6 \times 10^{-1}$  unit) were added to 2 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 7.5) containing 1.2 M maltose, and shaken at 160 oscillations per min at 40 °C for 36 h. Then, 0.4 ml of the reaction mixture was diluted with methanol up to 1.6 ml and filtered through a 0.2  $\mu$  m cellulose acetate membrane (Iwaki Glass Co., Ltd., Tokyo). The amounts of glucose, maltose, and  $\alpha$ -arbutin were measured by HPLC under conditions (A) and (B) described later in 4.2.6.

#### **4.2.4 Purification of hydroquinone glucoside**

The reaction mixture (4 ml) was extracted with ethylacetate (16 ml) to

remove hydroquinone. Following each extraction, the reaction mixture was centrifuged ( $2,000 \times g$ , 10 min, 4 °C) to clearly separate the organic and aqueous layers. Hydroquinone was extracted into the organic layer, and  $\alpha$ -arbutin and saccharides remained in the aqueous layer. The aqueous layer was then extracted with *n*-butanol (16 ml) instead of ethylacetate in the same way as described above. The *n*-butanol layer containing  $\alpha$ -arbutin was collected and dried using a rotary evaporator. The resulting precipitate was dissolved in 2 ml ethyl acetate-acetate-water (3:1:1, v/v/v), and loaded onto a silica column packed with Wakogel C-200 (Wako) with ethylacetate-acetate-water (3:1:1, v/v/v) as the eluent. Fractions containing  $\alpha$ -arbutin were detected by TLC according to the method shown in 4.2.5, collected, and dried using a rotary evaporator.

#### **4.2.5 Thin-layer chromatography (TLC)**

TLC was performed on silica gel 60 plates (E. Merck, Darmstadt, Germany) using the ascending method with ethylacetate-acetate-water (3:1:1, v/v/v) as the solvent. Spots were made visible by spraying with methanol-water- $\text{H}_2\text{SO}_4$  (40:17:3, v/v/v) followed by heating at 100 °C.

#### **4.2.6 High-performance liquid chromatography (HPLC)**

HPLC was performed using a Tosoh LC-8020 system with an Tosoh RI-8020. To detect and measure hydroquinone glucosides, the following conditions

(A) were used: column, TSK-Gel ODS 80-TS (4.6 × 250 mm, Tosoh Co., Tokyo); solvent, methanol-water (10:90, v/v); flow rate, 1.0 ml/min; and temperature, 40 °C. To detect and measure saccharides such as glucose and maltose, the following conditions (B) were used: column, Asahipak NH2P-50 4E (4.6 × 250 mm, Showa Denko. Co., Ltd., Tokyo); solvent, acetonitrile-10 mM tetra-*n*-propylammonium hydroxide containing acetic acid (pH 10.0) (70:30, v/v); flow rate, 1.0 ml/min; and temperature, 40 °C.

#### 4.2.7 NMR analysis

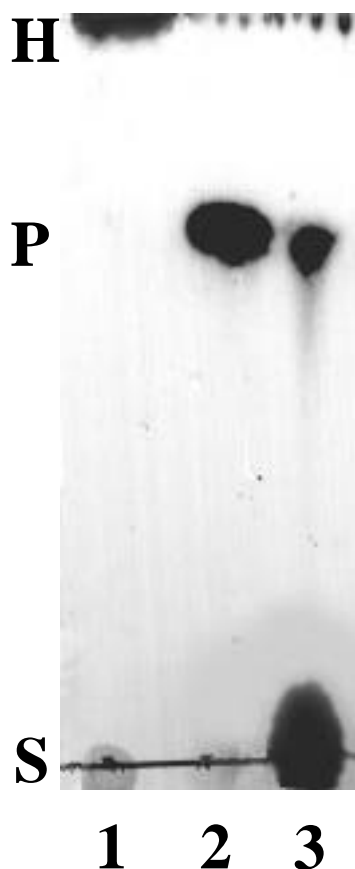
<sup>13</sup>C-NMR, <sup>1</sup>H-NMR and heteronuclear multiple bond coherence (HMBC) spectra were obtained using a JEOL JNM-LA 500 spectrometer (JEOL, Tokyo) operated at 125.65 MHz, 500 MHz, and 500 MHz, respectively, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard.

### 4.3 Results

#### 4.3.1 Preparation and isolation of *β*-arbutin

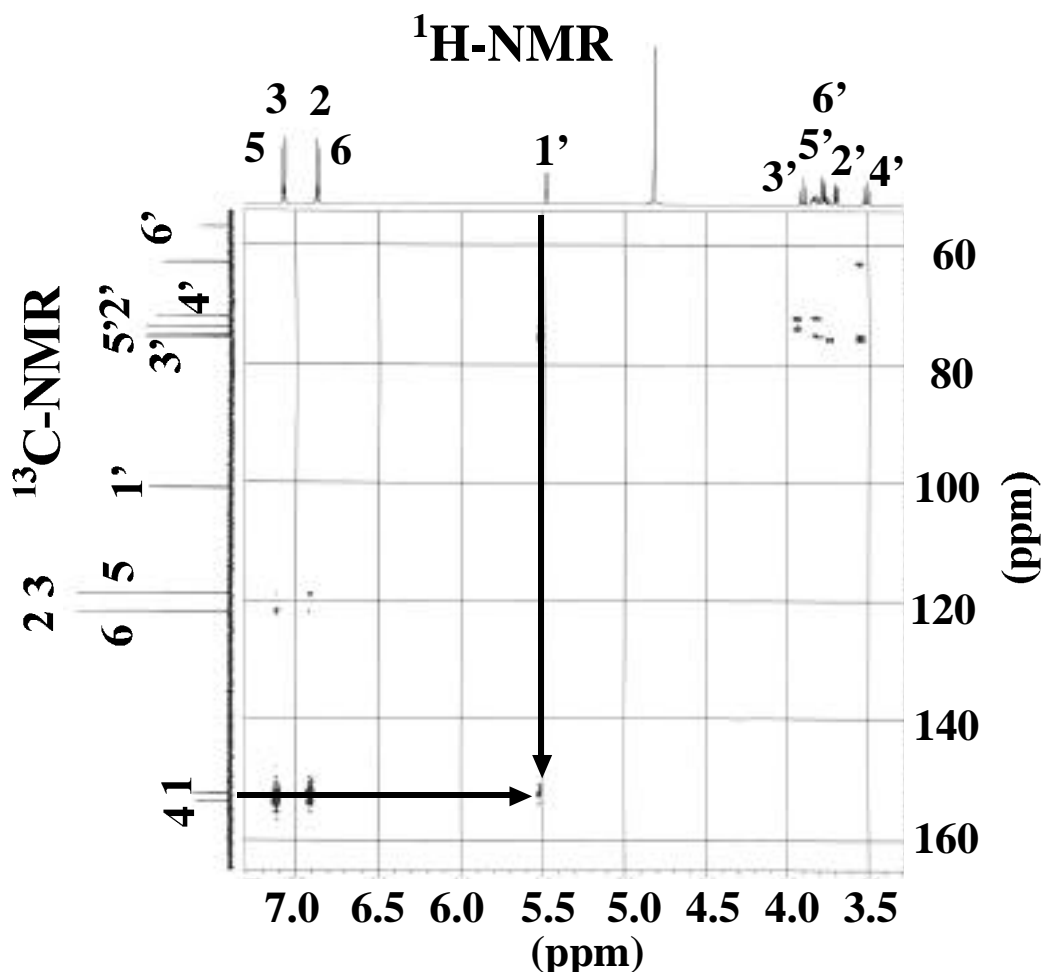
A typical TLC chromatogram of the reaction products is shown in Fig. 4.1. Since only one specific product was detected in the reaction mixture by TLC analysis. Since the product showed *R<sub>f</sub>* value of 0.66 identical to that of *β*-arbutin on TLC, it was presumed to be *β*-arbutin. Therefore, the author





**Fig. 4.1. TLC of the reaction products from hydroquinone and maltose with lyophilized cells of *X. campestris* WU-9701 as a biocatalyst. The reaction conditions are described in Materials and Methods. Abbreviations: H, hydroquinone; P, product (  $\alpha$ -arbutin); S, saccharides. Lanes: 1, standard hydroquinone; 2, standard  $\alpha$ -arbutin; 3, the reaction mixture. The  $R_f$  values of hydroquinone and the product (  $\alpha$ -arbutin) are 1.0 and 0.7, respectively.**

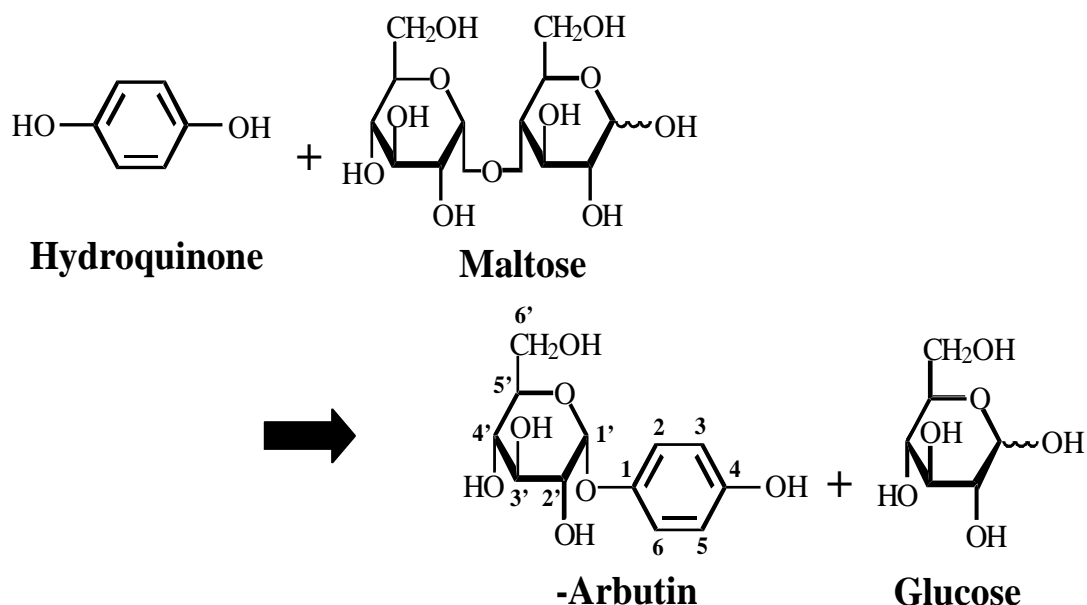
purified the product and subjected it to structure analyses. According to the method described in 4.2.4, the fractions containing  $\alpha$ -arbutin were selected, and the solid product was washed twice with 10 ml of water, and 15.1 mg of purified  $\alpha$ -arbutin was obtained. The molar conversion yield reached 30.5% based on



**Fig. 4.2. Two-dimensional HMBC (heteronuclear multiple bond coherence) spectrum of the isolated product.**

The ring numberings are identical to those shown in Fig. 4.1.  $^{13}\text{C}$ -NMR,  $^1\text{H}$ -NMR and HMBC spectra were obtained using a JEOL JNM-EX 600 NMR spectrometer operating at 600 Hz. Chemical shifts are expressed in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard.

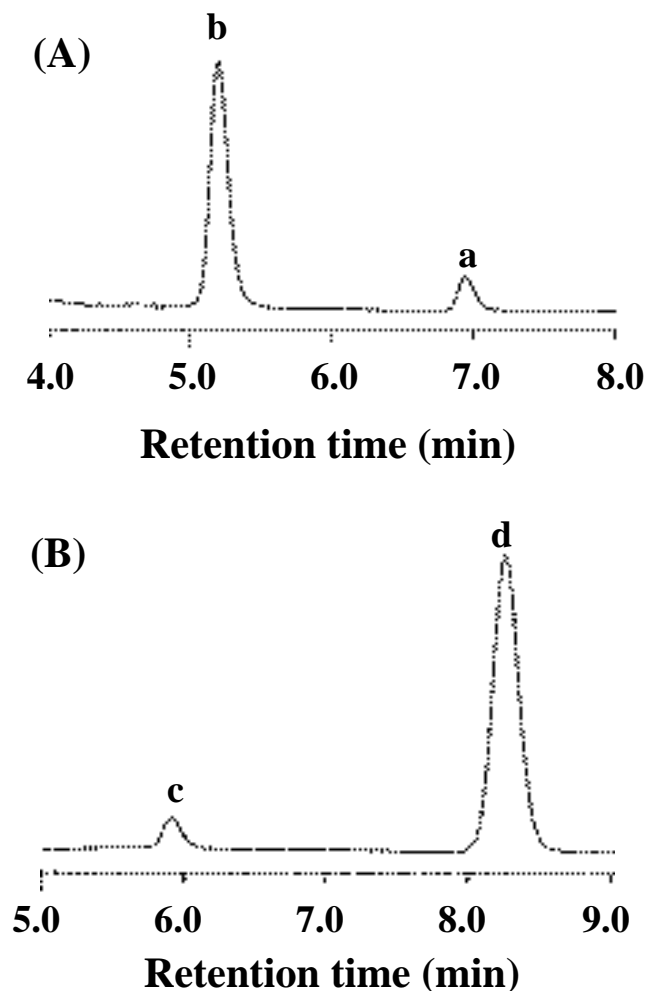
the amount of hydroquinone supplied. The  $^{13}\text{C}$ -NMR and  $^1\text{H}$ -NMR spectra of the isolated product were obtained, and the data suggest that the product consisted of hydroquinone and  $\beta$ -D-glucose (data not shown). A doublet signal at 5.5 ppm was assigned to the anomeric proton of the glucose moiety. This



**Fig. 4.3. Structure of hydroquinone 1- $\alpha$ -D-glucopyranoside (  $\alpha$ -arbutin) with ring numberings of carbons produced from maltose and hydroquinone by lyophilized cells of *X. campestris* WU-9701.**

signal had a smaller coupling constant ( $J=3.7$  Hz) than that for  $\beta$ -glucoside ( $J=7-9$  Hz). Moreover, to precisely determine the molecular structure of the product, a two-dimensional HMBC spectrum was obtained. As shown in Fig. 4.2, a sequence of correlation at the C1'/H1' position was clearly detected, indicating that  $\alpha$ -D-glucose was bonded to the C1 position of hydroquinone. Based on these results, the isolated product was identified as hydroquinone 1- $\alpha$ -D-glucopyranoside (  $\alpha$ -arbutin), and its structure is shown in Fig. 4.3.

HPLC chromatograms of the reaction mixture are shown in Fig. 4.4. In the analysis using an ODS column (Fig. 4.4A), a new peak corresponding to  $\alpha$ -arbutin at a retention time of 5.2 min was detected. In the analysis using an



**Fig. 4.4. HPLC of the reaction products from hydroquinone and maltose using lyophilized cells of *X. campestris* WU-9701. The reaction mixture was loaded onto (A) a TSK-Gel ODS-80TS column and (B) an Asahipak NH2P-50 4E column, details having been described in Materials and Methods. Peaks: a, hydroquinone; b,  $\alpha$ -arbutin; c, glucose; and d, maltose.**

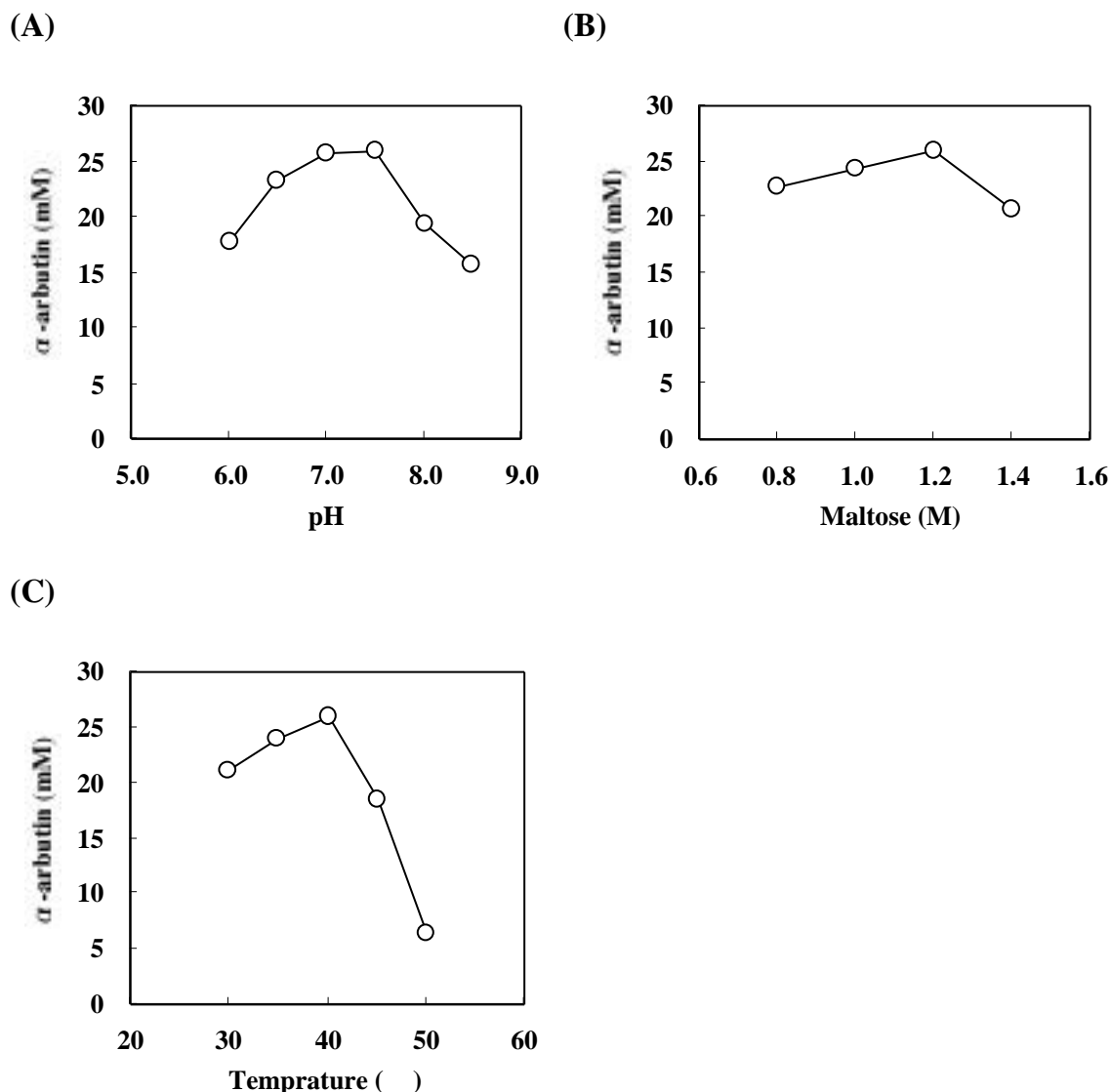
NH2P-50 4E column, glucose and maltose were detected at the retention times of 5.8 and 7.7 min, respectively (Fig. 4.4B). Other oligosaccharides, such as maltotriose, or other glucosylated derivatives, such as hydroquinone

oligoglucosides, were not detected, as were also observed for  $\alpha$ -MenG synthesis<sup>8)</sup>.

#### 4.3.2 Optimization of $\alpha$ -arbutin synthesis

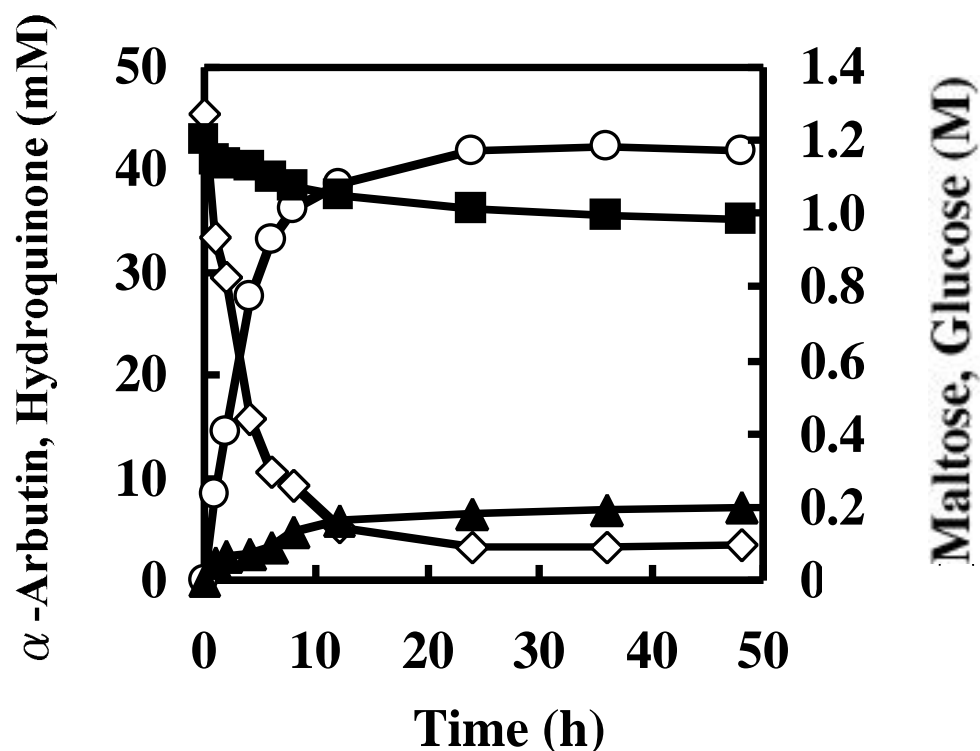
The optimum reaction conditions containing hydroquinone (45 mM) and lyophilized cells of *X. campestris* WU-9701 ( $6.6 \times 10^{-1}$  unit) were determined by sequentially changing the maltose concentration, pH, temperature. Figure 5A shows the effects of maltose concentration on the synthesis of  $\alpha$ -arbutin, and the amount of  $\alpha$ -arbutin produced was highest at 1.2 M. At the concentrations of maltose above 1.4 M, the production of  $\alpha$ -arbutin gradually decreased. As shown in Fig. 4.5B, the effects of pH on the synthesis of  $\alpha$ -arbutin were examined. The amount of  $\alpha$ -arbutin produced was highest at pH 7.5. As shown in Fig. 4.5C, the effects of temperature on the synthesis of  $\alpha$ -arbutin were examined. The amount of  $\alpha$ -arbutin produced was highest at 40 °C, but over 45 °C production of  $\alpha$ -arbutin decreased, probably due to inactivation of the enzyme caused by heat denaturation.

Moreover, the time course for the synthesis of  $\alpha$ -arbutin under the optimum conditions is shown in Fig. 4.6. The reaction for 36 h under the optimum conditions yielded 42 mM  $\alpha$ -arbutin, and the maximum molar conversion yield based on the amount of hydroquinone supplied reached 93%.



**Fig. 4.5. Effects of pH (A), maltose concentration (B) and temperature (C) on  $\alpha$ -arbutin synthesis by the lyophilized cells of *X. campestris* WU-9701.**

**(A)** Concentrations of maltose were changed in the standard reaction as described in Materials and Methods. **(B)** The pH of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer was changed to obtain the standard reaction conditions as described in Materials and Methods. **(C)** Temperature was changed in the standard reaction conditions as described in Materials and Methods.



**Fig. 4.6.** Time course of α-arbutin synthesis by the lyophilized cells of *X. campestris* WU-9701.

The reactions were carried out under the optimum conditions.

Symbols: ○, α-arbutin; ■, hydroquinone; ▲, glucose; ◇, maltose.

#### 4.4 Discussion

In this study, hydroquinone was efficiently and α-anomer-selectively glucosylated by the crude enzyme, showing α-glucosyl transfer activity, of *X. campestris* WU-9701. A maximum molar conversion yield of 93% was

achieved following a 36 h reaction under the optimum conditions.

In general, the enzymatic synthesis of glucoside is carried out via a transglucosylation reaction or the reverse hydrolysis reaction of glucosidases. Since lyophilized cells of *X. campestris* WU-9701 could not form  $\beta$ -arbutin when glucose instead of maltose was used as a carbohydrate substrate (data not shown), it seems likely that  $\beta$ -glucosylation of hydroquinone occurred via a transglucosylation reaction with maltose as  $\beta$ -glucose donor. In the transglucosylation reaction, a high concentration of the glucosyl donor is favorable, and in this study the optimum maltose concentration for  $\beta$ -arbutin production was 1.2 M as shown in Fig. 4.5A. The high transglucosylation activity of this enzyme may be useful not only for the efficient production of  $\beta$ -arbutin, but also for the synthesis of commercially important  $\beta$ -glucosides. Since other glucosylated products such as maltotriose or  $\beta$ -arbutin-G were not observed as shown in Fig. 4.4, there is the possibility that the enzyme prefers compounds having phenolic -OH groups, but not disaccharides, as acceptors.

Several researchers have reported on the enzymatic synthesis of  $\beta$ -arbutin using the purified enzyme<sup>3,4</sup>. However, the use of the purified enzyme is generally expensive and seems difficult for bulky products on an industrial scale. On the other hand, the crude enzyme of *X. campestris* was prepared using a simple method as described in Materials and Methods, and it should be easy and cheap to use this method for the large-scale production of  $\beta$ -arbutin. Since the reaction mixture used in the present study contains no components harmful to the human body, the reaction system using the lyophilized cells of *X. campestris* WU-9701 described in this study seems to be applicable for the



production of  $\alpha$ -arbutin as a cosmetic additive on an industrial scale. Moreover, this system might be useful for the  $\beta$ -anomer-selective glucosylation of other polyphenols or phenolic compounds.

It is interesting to note that only one -OH group in the hydroquinone was glucosylated through the reaction although hydroquinone has two phenolic -OH groups at *para* position in its structure. The author also detected only one glucosylated product for each regio-isomer of hydroquinone, catechol (*ortho*-isomer) and resorcinol (*meta*-isomer) (data not shown). Such unique properties enable the synthesis of  $\alpha$ -arbutin with a high conversion yield of 93%, based on the amount of hydroquinone supplied. To our knowledge, this molar conversion yield, 93%, is the highest among the data reported by several researchers<sup>3,4)</sup>, who used the purified enzyme but have not succeeded in selective synthesis of  $\alpha$ -arbutin. Since the lyophilized cells of *X. campestris* WU-9701 can be prepared using a simple method, it should be easy and inexpensive to use the method described in this chapter for large-scale production of  $\alpha$ -arbutin.

In conclusion, the author succeeded in establishing a simple and efficient method for the  $\beta$ -anomer-selective synthesis of  $\alpha$ -arbutin. To our knowledge, the molar conversion yield of 93%, based on the amount of hydroquinone supplied, is the highest reported to date.

## 4.5 Reference

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## Chapter 5 Purification and Characterization of a Novel -Glucosyl Transfer Enzyme from *Xanthomonas campestris* WU-9701

### 5.1 Introduction

The author described that the enzyme of *X. campestris* WU-9701 was available for (+)-catechin 3'-*O*- $\beta$ -D-glucopyranoside ( $\beta$ -C-G) synthesis by  $\beta$ -anomer- and regio-selective glucosylation, and that  $\beta$ -C-G was synthesized with 57% of a molar conversion yield in Chap. 3. Since only one -OH group at 3' position in the (+)-catechin molecule, having five -OH groups, was selectively  $\beta$ -glucosylated through the reaction, this enzyme seems to possess a high regioselectivity toward a substrate as an  $\beta$ -glucosyl acceptor. The author also described that this enzyme was available for  $\beta$ -anomer-selective glucosylation of hydroquinone to produce hydroquinone  $\beta$ -glucoside ( $\beta$ -arbutin) with 93% of a molar conversion yield in Chap. 4. In the reaction as well as the  $\beta$ -C-G synthesis reaction, by using the enzyme of *X. campestris* WU-9701, maltooligosaccharides such as maltotriose and maltotetraose were not synthesized. From these results, the author considered that this enzyme was different from the usual  $\beta$ -glucosidase and decided to clarify the properties of the purified enzyme for characterization.

In this chapter, the author describe the purification and characterization of

this novel “ $\alpha$ -glucosyl transfer enzyme”, catalyzing  $\alpha$ -anomer-selective glucosylation, produced by *X. campestris* WU-9701. This is the first report as for such an enzyme having low hydrolyzing activity toward maltose but high  $\alpha$ -glucosyl transfer activity toward -OH groups of phenolic and alcoholic molecules but not to -OH groups of saccharides.

## **5.2 Materials and Methods**

### **5.2.1 Strains and materials**

*X. campestris* WU-9701, a producer of  $\alpha$ -glucosyl transfer enzyme, was used as a parental strain. *l*-Menthol and hydroquinone were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Authentic  $\alpha$ -MenG was synthesized by the enzymatic reaction using lyophilized cells of *X. campestris* WU-9701, purified, and used as a standard. All other chemicals used were commercially available and of a chemically pure grade.

### **5.2.2 Enzyme assay**

$\alpha$ -Glucosyl transfer activity was measured using maltose and hydroquinone as substrates as described in Chap. 2. A portion of 0.4 ml of the crude enzyme solution and 45 mM hydroquinone were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer containing 1.5 M maltose (pH 8.5) to give finally

1.2 M maltose in 2 ml of the reaction mixture, and incubated by shaking at 180 rpm at 40°C for 60 min. The reaction was stopped by heating in the boiling water at 100°C for 10 min. The amount of hydroquinone  $\alpha$ -glucoside formed was measured using high-performance liquid chromatography (HPLC), as described in Chap. 2. One unit (U) of  $\alpha$ -glucosyl transfer activity was defined as the amount of enzyme that produces one  $\mu$  mole of hydroquinone  $\alpha$ -glucoside per minute from hydroquinone under the conditions described above. When maltose hydrolysis activity was measured, the same conditions for  $\alpha$ -glucosyl transfer activity described above were used with the exception that hydroquinone was omitted. The amount of maltose was measured using HPLC as described in Chap. 2.

### 5.2.3 Purification of $\alpha$ -glucosyl transfer enzyme

Cells of *X. campestris* WU-9701 were grown under aerobic conditions with shaking at 30°C for 48 h in one liter of medium containing (per liter) 50 g maltose, 2.0 g yeast extract (Difco, U.S.A.), 10 g peptone, and 1.0 g MgSO<sub>4</sub>. The pH was initially adjusted to 7.0 with 2.0 M NaOH. After 48 h cultivation, cells were harvested by centrifugation (15,000  $\times$  g, 30 min, 4°C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0).

Purification steps were carried out at 0-4°C unless otherwise indicated. Harvested cells were suspended in 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0, A-buffer) 50 ml, disrupted by sonication (20 kHz, 200 W, 120 min, 0°C), and centrifuged (25,000  $\times$  g, 60 min, 4°C) again. The resulting supernatant 50

ml was collected as the cell-free extract. Then, ammonium sulfate powder was added to give 30% (w/v) saturation, and the solution was stirred at 4°C over night. The resulting precipitate was removed by centrifugation (25,000 × g, 60 min, 4°C). Furthermore, the supernatant was adjusted to 60% (w/v) saturation with ammonium sulfate, and stirred at 4°C for over night. The resulting precipitate was collected by centrifugation (25,000 × g, 60 min, 4°C) and dissolved in 30 ml of A-buffer. This solution was dialyzed against A-buffer over night.

The dialysate was applied to a DEAE-Toyopearl 650S column (2.5 × 6 cm; Tosoh Co., Tokyo) that was loaded with 60 ml of A-buffer containing 1 M NaCl and equilibrated with A-buffer. Elution was carried out with a continuous linear gradient of 0 to 0.3 M NaCl in 240 ml of A-buffer. Active fractions were collected and concentrated by ultrafiltration. The concentrated eluate was put on a Superdex 200 column (1.6 × 60 cm; Amersham Biosciences, NJ, USA) equilibrated with A-buffer containing 0.15 M NaCl. Active fractions were collected, dialyzed against A-buffer, concentrated by ultrafiltration, and then applied to a maltose-agarose column (1 × 3.5 cm; Sigma Chemicals Co., St. Louis, USA) that was loaded with 30 ml of A-buffer containing 1 M NaCl and equilibrated with A-buffer. Elution was carried out with a continuous linear gradient of 0 to 0.4 M NaCl in 200 ml of A-buffer. Active fractions were collected, dialyzed against A-buffer over night, concentrated by ultrafiltration, and then applied to a Q-Sepharose column (1.6 × 10 cm; Amersham Biosciences) that was loaded with 60 ml of A-buffer containing 1 M NaCl equilibrated with A-buffer containing 0.1 M NaCl. Elution was carried out with

a continuous linear gradient of 0.1 to 0.3 M NaCl in 200 ml of A-buffer. Active fractions were collected, dialyzed against A-buffer over night, concentrated by ultrafiltration, and then applied to the same column that was loaded with 60 ml of A-buffer containing 1 M NaCl equilibrated with A-buffer containing 0.15 M NaCl. Elution was carried out with a continuous linear gradient of 0.15 to 0.3 M NaCl in 200 ml of A-buffer. Active fractions were collected and stored at 4°C after dialysis against A-buffer, for the characterization of the enzyme.

#### **5.2.4 Characterization of the purified enzyme**

Effects of metal ions and some reagents on the enzyme were examined under the standard assay conditions, except for the addition of each reagent dissolved in 10 mM NH<sub>3</sub>-NH<sub>4</sub>Cl buffer (pH 8.5) to give a final concentration of 10 mM in the reaction mixture.

#### **5.2.5 Other analytical methods**

Protein concentrations were determined by the method of Bradford<sup>1)</sup> using the Coomassie Protein Assay Kit (Pierce Chem. Co., Rockford, USA) with bovine serum albumin as a standard. For column chromatography, the protein concentration was measured by the absorbance at 280 nm using a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using 10% (w/v) polyacrylamide by the method of Laemmli<sup>2)</sup>.



HPLC was done with a Shimadzu LC-6A system with RID-6A differential refractometer (Shimadzu). To detect and measure  $\alpha$ -MenG and  $\alpha$ -arbutin, the same conditions as described in Chap. 2 were used.

## 5.3 Results

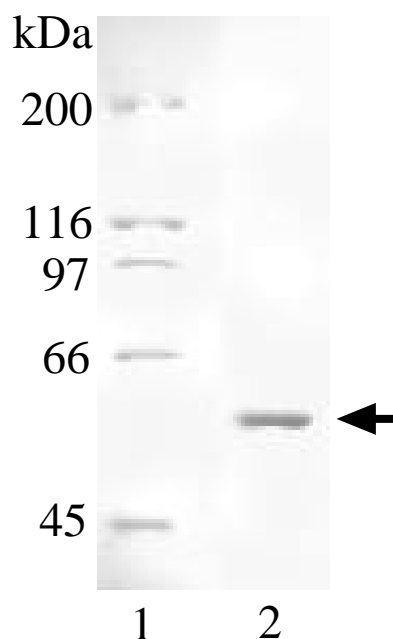
### 5.3.1 Purification of the $\alpha$ -glucosyl transfer enzyme

*X. campestris* WU-9701 produced an unique glucose transfer enzyme, catalyzing  $\alpha$ -anomer-selective glucosylation. For 24-h and 48-h cells of *X. campestris* WU-9701, more than 90% of the total activity of the enzyme was confirmed to be localized in the cytosol fruction (data not shown). Therefore, the  $\alpha$ -glucosyl transfer enzyme from *X. campestris* WU-9701 was purified from the cell-free extract as described in Materials and Methods. Purification steps are shown in Table 5.1. The enzyme was purified 99.6-fold with a yield of 0.33%, and its final specific activity was 3.46 U/mg. As shown in Fig. 5.1, the purified enzyme produced a single band on the gel by SDS-PAGE and its molecular weight was estimated to be 57 kDa. On the other hand, the molecular weight was calculated to be 60 kDa by Superdex 200 gel filtration. Since the molecular weights of the enzyme measured by the two methods were approximately the same, the enzyme was considered to be monomeric one.

Table 5.1. Purification steps of the  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701.

Step	Total protein (mg)	Specific activity <sup>a)</sup> (unit/mg)	Yield (%)	Purification (fold)
Crude enzyme	$4.78 \times 10^2$	$3.47 \times 10^{-2}$	$1.00 \times 10^2$	1.00
Ammonium sulfate precipitation (30-60%)	$2.58 \times 10^2$	$4.02 \times 10^{-2}$	$6.30 \times 10$	1.16
DEAE-Toyopearl 650S (Anion-exchange)	$2.30 \times 10$	$2.02 \times 10^{-1}$	$2.80 \times 10$	5.83
Superdex 200 (Gel filtration)	5.19	$4.47 \times 10^{-1}$	$1.40 \times 10$	$1.29 \times 10$
Maltose-agarose (Affinity)	$8.87 \times 10^{-1}$	1.31	6.98	$3.77 \times 10$
1 <sup>st</sup> Q-Sepharose (Anion-exchange)	$3.17 \times 10^{-2}$	3.40	$6.56 \times 10^{-1}$	$9.82 \times 10$
2 <sup>nd</sup> Q-Sepharose (Anion-exchange)	$1.60 \times 10^{-2}$	3.46	$3.30 \times 10^{-1}$	$9.96 \times 10$

<sup>a)</sup>The enzyme activity was estimated as glucose transfer activity of was measured using hydroquinone as a substrate. One unit of  $\alpha$ -glucosyl transfer activity was defined as the amount of enzyme that produces one  $\mu$  mole of hydroquinone  $\alpha$ -glucoside per minute from hydroquinone under the conditions described in Materials and Methods.



**Fig. 5.1. SDS-PAGE of the purified  $\alpha$ -glucosyl transfer enzyme from *X. campestris* WU-9701.**

**The protein was stained with Coomassie brilliant blue R-250.**

**Myosin (200 kDa),  $\alpha$ -galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa) were used as molecular marker standards. Lanes: 1, molecular weight standards; 2, Purified enzyme. The arrow indicates 57 kDa for the molecular weight of  $\alpha$ -glucosyl transfer enzyme.**

### 5.3.2 Effects of various reagents

Effects of various metal ions and chemical reagents on  $\alpha$ -arbutin synthesis activity of the  $\alpha$ -glucosyl transfer enzyme are shown in Table 5.2. The enzyme activity was strongly inhibited by bivalent metal cations such as  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Zn}^{2+}$ . Since  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$  and *p*CMB reduced the enzyme activity, it seems likely

Table 5.2. Effects of various metal ions on -arbutin synthesis activity of the purified enzyme.

Reagents	Relative activity (%)
None	100
KCl	194
LiCl	149
NaCl	181
CaCl <sub>2</sub>	81
CuCl <sub>2</sub>	0
HgCl <sub>2</sub>	0
MgCl <sub>2</sub>	56
MnCl <sub>2</sub>	29
ZnCl <sub>2</sub>	0
<i>p</i> CMB*	0

\**p*-Chloromercuribenzoic acid.

Effects of various reagents were examined under the standard assay conditions with the addition of various reagents at final concentration of 10 mM, in 10 mM NH<sub>3</sub>-NH<sub>4</sub>Cl buffer (pH 8.5).

that sulfhydryl groups may be involved at its active site. The enzyme activity was slightly activated by K<sup>+</sup> and Na<sup>+</sup>.

### 5.3.3 Substrate specificity

To confirm the hydrolyzing activity toward saccharides containing glucose

Table 5.3. Hydrolysis reaction by the purified enzyme toward various saccharides.

Saccharide	Concentration	Hydrolysis (mM)
Trehalose	100 mM	0
Kojibiose	100 mM	0
Nigerose	100 mM	4.71
Maltose	100 mM	$1.99 \times 10$
Isomaltose	100 mM	$3.10 \times 10^{-1}$
Cellobiose	100 mM	0
Maltotriose	100 mM	$3.81 \times 10^{-1}$
Maltotetraose	100 mM	Trace
Amylose	3.6% (w/v)	$6.44 \times 10^{-1}$
<i>p</i> NPG*	25 mM	$1.75 \times 10^{-1}$
Sucrose	100 mM	0

\**p*-Nitrophenyl -D-glucopyranoside

Hydrolysis reaction was examined under the standard assay conditions with the addition of various saccharides at final concentration described in Materials and Methods, in 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 8.5).

molecule, the -glucosyl transfer enzyme was incubated with various saccharides, and amounts of glucose liberated were measured (Table 5.3). Although hydrolyzing activity was relatively low as described later, among the reactions tested the enzyme activity toward maltose was the highest, and slightly hydrolyzed nigerose. The enzyme did not hydrolyze sucrose. Moreover, this enzyme hydrolyzed slightly maltotriose and maltotetraose although they have -1, 4 linkage of glucose in their molecules as well as maltose.

Table 5.4.  $\alpha$ -Glucosyl transfer reaction toward hydroquinone by the purified enzyme using various saccharides as  $\alpha$ -glucosyl donors.

Saccharide	Concentration	$\alpha$ -Arbutin (mM)
Trehalose	1.2 M	0
Kojibiose	1.2 M	0
Nigerose	1.2 M	0
Maltose	1.2 M	$2.5 \times 10$
Isomaltose	1.2 M	0
Cellobiose	300 mM	0
Maltotriose	300 mM	0
Maltotetraose	300 mM	0
Amylose	3.6% (w/v)	0
<i>p</i> NPG*	300 mM	0
Sucrose	1.2 M	0

\**p*-Nitrophenyl  $\alpha$ -D-glucopyranoside

$\alpha$ -Glucosyl transfer reaction was examined under the standard assay conditions with the addition of various saccharides at a final concentration described in Materials and Methods, in 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 8.5).

To confirm the  $\alpha$ -glucosyl transfer activity using saccharides containing glucose molecule as  $\alpha$ -glucosyl donors, the  $\alpha$ -glucosyl transfer enzyme was incubated with various saccharides and hydroquinone, and amounts of  $\alpha$ -arbutin formed were measured. As shown in Table 5.4, this enzyme produced  $\alpha$ -arbutin using hydroquinone and maltose, but not any other saccharides. Among the saccharides tested, only maltose having  $\alpha$ -1, 4 linkage

of glucose was utilized as the  $\alpha$ -glucosyl donor for the enzyme, suggesting that this enzyme possesses a high substrate specificity toward a substrate as an  $\alpha$ -glucosyl donor.

#### 5.3.4 Kinetic properties

Kinetic constants of  $\alpha$ -glucosyl transfer enzyme toward maltose were determined as shown in Table 5.5 from Lineweaver-Burk plots. The  $K_m$  value of  $\alpha$ -glucosyl transfer enzyme for maltose in the presence of hydroquinone as an acceptor was determined to be  $4.4 \times 10^2$  mM and was approximately  $2.0 \times 10$ -fold higher than that in the absence of hydroquinone,  $2.1 \times 10$  mM. On the other hand,  $V_{max}$  and  $K_{cat}$  of  $\alpha$ -glucosyl transfer enzyme for maltose in the presence of hydroquinone were higher than those in the absence of hydroquinone. In the presence of hydroquinone and maltose as substrates,  $\alpha$ -glucosyl transfer reaction was mainly occurred without hydrolysis of maltose and produced glucose of molecule equivalent to the molecule of maltose consumed. On the other hand, in the absence of hydroquinone, only maltose-hydrolysis reaction was occurred and produced glucose of the molecule equivalent to 2 times molecules of maltose consumed.

As shown in Fig. 4.6 in Chap. 4, in the time course of  $\alpha$ -arbutin synthesis amounts of  $\alpha$ -arbutin and glucose were almost the same during the  $\alpha$ -glucosyl transfer reaction using the  $\alpha$ -glucosyl transfer enzyme. In addition,  $V_{max}$  and turn over ( $k_{cat}$ ) of the  $\alpha$ -glucosyl transfer enzyme in the presence of hydroquinone were higher than those in the absence of hydroquinone. These

Table 5.5. Kinetic constants of the purified  $\alpha$ -glucosyl transfer enzyme.

Hydroquinone	$K_m$ (mM)	$V_{max}$ (mM/sec)	$k_{cat}$ (sec <sup>-1</sup> )	$k_{cat}/K_m$ (sec <sup>-1</sup> • mM <sup>-1</sup> )
None	$2.1 \times 10^2$	$3.3 \times 10^{-3}$	$4.7 \times 10^2$	$2.2 \times 10^{-5}$
Added (45 mM)	$4.4 \times 10^2$	$5.4 \times 10^{-3}$	$7.7 \times 10^2$	1.7

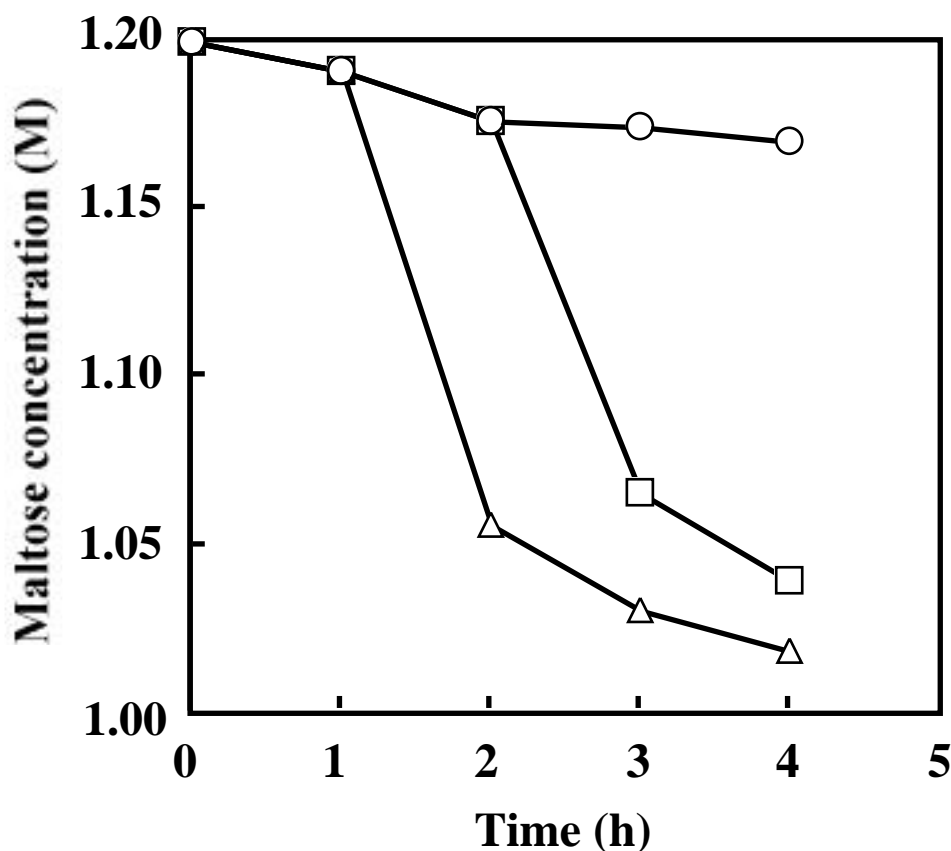
Hydroquinone 10 mg and 0.1 ml of purified  $\alpha$ -glucosyl transfer enzyme ( $9.48 \times 10^{-5}$  U) were added to 0.9 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 8.5) containing various concentrations (from 15 to 1500 mM) of maltose, and shaken at 180 rpm at 40 °C for 3 min. The amounts of maltose and hydroquinone  $\alpha$ -glucoside were measured by HPLC under the conditions as described in Materials and Methods. Glucose transfer and maltose hydrolysis activities were determined as described in Materials and Methods.

results suggested that the  $\alpha$ -glucosyl transfer reaction proceeded predominantly in comparison to the maltose-hydrolysis reaction by this enzyme.

### 5.3.5 Maltose-hydrolyzing and glucose transfer activity

Maltose-hydrolyzing and  $\alpha$ -glucosyl transfer activities of the enzyme were investigated using hydroquinone as a substrate, as shown in Fig. 5.3. When hydroquinone was not added to the reaction mixture, rate of decrease of maltose was very slow. On the contrary, hydroquinone was added after one or two hours after starting the reaction, decrease of maltose was rapidly accelerated. These results suggested that the  $\alpha$ -glucosyl transfer enzyme catalyzed fastly the





**Fig. 5.3. Time course of  $\alpha$ -glucosyl transfer and hydrolysis reactions using purified  $\alpha$ -glucosyl transfer enzyme.**

The reaction mixture, containing the purified enzyme 0.1 ml ( $9.48 \times 10^{-5}$  U) and 0.9 ml of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer (pH 8.5) containing 1.2 M maltose, was shaken at 180 rpm at  $40^\circ\text{C}$ . Then, hydroquinone 10 mg was added to the reaction mixture after 1 h or 2 h incubation. The amount of maltose, by every 1 h, was measured by HPLC under the conditions (B) as described in Materials and Methods. Symbols:  $\circ$ , without hydroquinone;  $\square$ , hydroquinone added after 1 h incubation;  $\triangle$ , hydroquinone added after 2 h incubation.

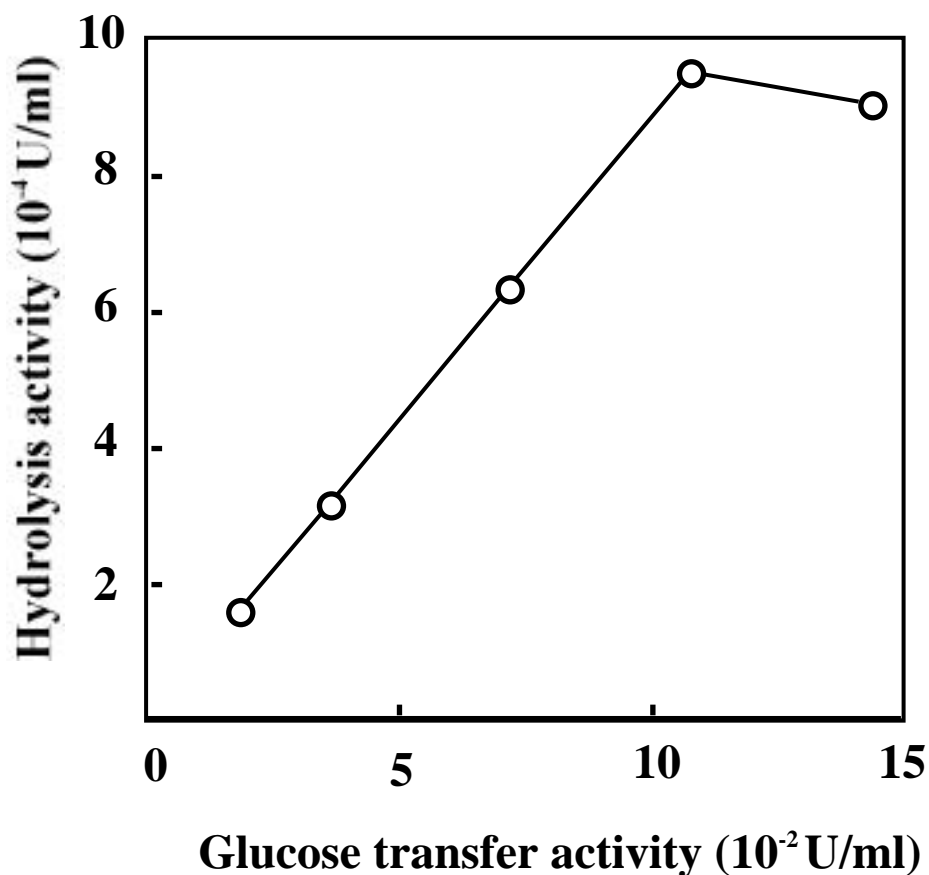
$\alpha$ -glucosyl transfer reaction in the presence of acceptor molecules such as hydroquinone but slowly the maltose-hydrolysis reaction regardless of the

presence or absence of acceptor molecules.

## 5.4 Discussion

In this chapter, the author described that  $\alpha$ -glucosyl transfer enzyme, the enzyme catalyzing  $\alpha$ -anomer-selective glucosylation, was purified to homogeneity from a cell-free extract of the *X. campestris* WU-9701, and its properties were investigated. The enzyme was purified 99.6-fold with a yield of 0.33% (Table 5.1). Since the molecular weights determined by SDS-PAGE, under fully dissociating conditions, and determined by gel filtration chromatography on Superdex 200, under native conditions, are in good agreement (57 kDa and 60 kDa, respectively), the enzyme was considered to be a monomeric enzyme.

To confirm the properties of the  $\alpha$ -glucosyl transfer enzyme, effects of addition of hydroquinone as an  $\alpha$ -glucosyl acceptor to the reaction mixture containing maltose as an  $\alpha$ -glucosyl donor. In the absence of hydroquinone, maltose reduction was little, but in the presence of hydroquinone,  $\alpha$ -glucosyl transfer reaction was actively occurred and the amount of maltose was rapidly decreased (Table 5.5 and Fig. 5.3). On the other hand, as shown in Table 5.4, among the saccharides tested only maltose was utilized as the  $\alpha$ -glucosyl donor. These results suggested that the enzyme possesses a high substrate specificity toward a substrate as an  $\alpha$ -glucosyl donor and that other disaccharides or trisaccharides are not suitable for the glucosyl transfer reaction.



**Fig. 5.4. Relation of glucose transfer activity and hydrolysis activity of the purified  $\alpha$ -glucosyl transfer enzyme.**

Hydroquinone 10 mg and various amounts of purified enzyme were added to 0.9 ml of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer (pH 8.5) containing 1.2 M maltose, and shaken at 180 rpm at  $40^\circ\text{C}$  for 24 h.

The amounts of glucose, maltose, and  $\alpha$ -arbutin were measured by HPLC under the conditions as described in Chap. 2.

Kinetic constants of  $\alpha$ -glucosyl transfer enzyme toward maltose were determined as shown in Table 5.5. The  $V_{\text{max}}$  and  $k_{\text{cat}}$  values for maltose in the presence of hydroquinone were higher than those in the absence of hydroquinone. As shown in Fig. 4.6 in Chap. 4, in the time course of  $\alpha$ -arbutin

synthesis, the amounts of  $\alpha$ -arbutin and glucose were almost the same after the glucosylation reaction using  $\alpha$ -glucosyl transfer enzyme after 10 min from the beginning of the reaction. These results suggest that the  $\alpha$ -glucosyl transfer enzyme catalyzed fastly the  $\alpha$ -glucosyl transfer reaction in the presence of hydroquinone (an acceptor) but slowly the maltose-hydrolysis reaction regardless of the presence or absence hydroquinone and and that the enzyme is not usual  $\alpha$ -glucosidase mainly catalyzing hydrolysis reaction. Such a property contributed to keep selective and efficient  $\alpha$ -glucoside synthesis of  $\alpha$ -glucosides such as  $\alpha$ -C-G and  $\alpha$ -arbutin. These properties are very unique and not found for the reactions with the  $\alpha$ -glucosidase of *Saccharomyces cerevisiae*<sup>3)</sup> or  $\alpha$ -amylase of *Bacillus subtilis*<sup>4)</sup>, and enabled us to synthesize  $\alpha$ -glucoside with the high conversion yield.

## 5.5 References

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## Chapter 6 Gene Cloning and Molecular Characterization of a Novel $\alpha$ -Glucosyl Transfer Enzyme from *Xanthomonas campestris* WU-9701

### 6.1 Introduction

In Chap. 5, the author described the purification and characterization of the novel  $\alpha$ -glucosyl transfer enzyme from *X. campestris* WU-9701, catalyzing  $\alpha$ -anomer-selective glucosylation with maltose as a  $\alpha$ -glucosyl donor. In this chapter, the author describes that, in order to determine the primary structure of this enzyme, the gene (*xgtA*) encoding the  $\alpha$ -glucosyl transfer enzyme was cloned and identified. The gene *xgtA* was expressed under the control of *lac* promoter in *Escherichia coli* JM109. Based on the deduced amino acid sequence of XgtA, its secondary structure is putatively determined by DDBJ-SS-Thread program of DDBJ. Moreover, 3D structure of XgtA is predicted and drawn from the primary structure by computer program 3D-JIGSAW program of ICRF.

### 6.2 Materials and Methods

#### 6.2.1 Strains and plasmids

*X. campestris* WU-9701, a producer of  $\alpha$ -glucosyl transfer enzyme, was

used as a parental strain. *E. coli* JM109, as described in Chap. 2, was used as a host for the construction of a DNA library and subcloning of DNA fragments. A plasmid pUC18 (Maxim Biotech., Inc., Canada) was used as a vector.

### **6.2.2 Enzyme assay**

Cells of recombinant *E. coli* such as JM109/pUGTF-7 were grown under aerobic conditions with shaking at 37°C for 22 h in one liter of LB medium containing 100 mg/ml of ampicillin and 0.8 mM IPTG. The pH was initially adjusted to 7.0 with 2.0 M NaOH. After 22 h cultivation, cells were harvested by centrifugation (6,000 × g, 15 min, 4°C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). The cells were resuspended in 5 ml of 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0), disrupted by sonication (20 kHz, 200 W, 10 min), and centrifuged (20,000 × g, 30 min, 0°C). The resulting supernatant 5 ml was collected as the cell-free extract.

Enzyme assay was performed as described in Chap. 2. -Glucosyl transfer activity was measured using maltose and hydroquinone as substrates as described in Chap. 2. A portion of 0.4 ml of the cell-free extract and 45 mM hydroquinone were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer containing 1.5 M maltose (pH 8.5) to give finally 1.2 M maltose in 2 ml of the reaction mixture, and incubated by shaking at 180 rpm at 40°C for 60 min. The reaction was stopped by heating in the boiling water at 100°C for 10 min. The amount of hydroquinone -glucoside formed was measured using high-performance liquid chromatography (HPLC), as described in Chap. 2. One unit

(U) of  $\alpha$ -glucosyl transfer activity was defined as the amount of enzyme that produces one  $\mu$  mole of hydroquinone  $\alpha$ -glucoside per minute from hydroquinone under the conditions described above. When maltose hydrolysis activity was measured, the same conditions for  $\alpha$ -glucosyl transfer activity described above were used with the exception that hydroquinone was omitted.

### 6.2.3 Amplification by the polymerase chain reaction (PCR)

To obtain a gene fragment partially encoding  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701, PCR was performed with a total DNA of *X. campestris* WU-9701 as a template. First, two oligonucleotide primers for use in the partial amplification of *xgtA* by PCR were designed with reference to the N-terminal and internal amino acid sequences of the purified enzyme. The structures of degenerate primers specific for the 5'-converted region were 5'-CARACICCTGGTGGMG-3' and those specific for the internal region were 5'-AGIACYTGRTCKATCAT-3', where I, R, M, Y and K show deoxyinosine, A+G, A+C, C+T and G+T, respectively.

Total DNA (4  $\mu$ g) purified from *X. campestris* WU-9701 was used as a template in a 500  $\mu$ l of reaction mixture with 12.5 units of Taq polymerase (Nippon Roche, Tokyo, Japan). The amplification conditions were 95°C for 3 min at the start, then 95°C for 60 s, 49-56°C for 60 s, and 72°C for 150 s for total of 30 cycles. The PCR product was used as a probe for screening the corresponding gene in the gene library of *X. campestris* WU-9701.



#### **6.2.4 Construction of a partial DNA library of *X. campestris* WU-9701 and screening**

Total DNA of *X. campestris* WU-9701 was digested with restriction enzyme *SalI* and then separated by 1.0% (w/v) agarose gel electrophoresis in 40 mM Tris-acetate-1 mM EDTA buffer (pH 8.0) at 100 V for 30 min. The digested 4.0- to 7.0-kb DNA fragments were cut out from the agarose gel and purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). A calf interstitial alkaline phosphatase-treated and *SalI*-digested pUC18 vector was ligated with digested DNA fragments of *X. campestris* WU-9701. The resulting plasmids were used for transformation of *E. coli* JM109 by electroporation using 0.1 cm cell (1800 V, 400 W, 25 mF) on a Gene Pulser (Nippon Bio-Rad, Tokyo, Japan). The recombinants, growing as white colonies on LB agar supplemented with ampicillin, X-Gal and IPTG, were screened by colony hybridization using a Hybond-N<sup>+</sup> membrane (Amersham Biosciences) with a probe of the PCR product as described above, and labeled with DIG-dUTP (Roche Diagnostics, Mannheim, Germany) under the stringent condition of 68°C.

#### **6.2.5 Sequencing of the nucleotides**

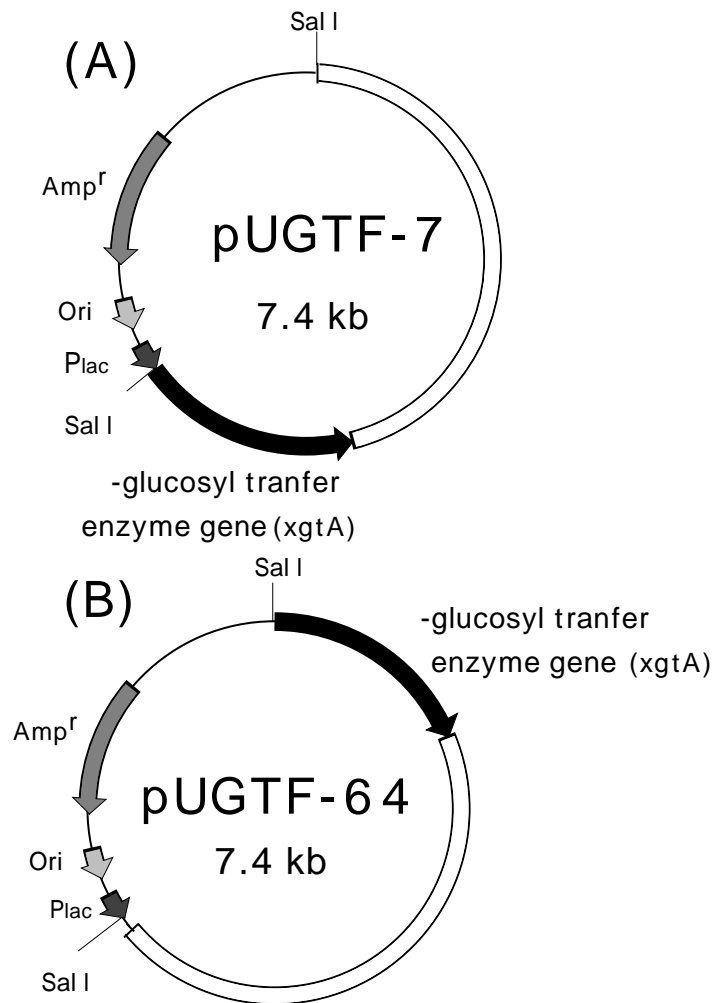
The insert DNA of recombinant plasmid was sequenced by the ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Japan, Tokyo, Japan) in accordance with the manufacture's instructions. An automatic ABI Prism 310 sequencer (Applied Biosystems

Japan) was used for determination of nucleotide sequences. Based on the information as for the insert DNA sequence, new sequencing primers were designed from the sequence data until all of the insert sequence had been sequenced from both strands. The nucleotide and deduced amino acid sequences were analyzed with the aid of the computer program GENETYX-MAC ver. 11.2.0 (Software Development Co. Ltd., Tokyo, Japan). The Genbank and Swissprot databases were searched for the similarities of nucleic acid and amino acid using FASTA program of the DNA Data Bank of Japan (DDBJ) and BLAST program of the National Center for Biotechnology Information (NCBI). The putative secondary structure of XgtA was searched using SS-Thread program of DDBJ. The Protein Data Bank database were searched for the selection of model and the drawing of molecular structure of XgtA using LIBRA of DDBJ and 3D-JIGSAW program of ICRF (Imperial Cancer Research Fund, London, UK), respectively.

## **6.3 Results**

### **6.3.1 Cloning of the gene (*xgtA*) encoding an $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701**

As for the purified  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701, the amino acid sequences corresponding to N-terminal (SQTPWWRGAVIYQIYPRSFL) and internal (VMIDQVLSHTSI) sequences



**Fig. 6.1. Physical maps of (A) pUGTF-7 and (B) pUGTF-64.**

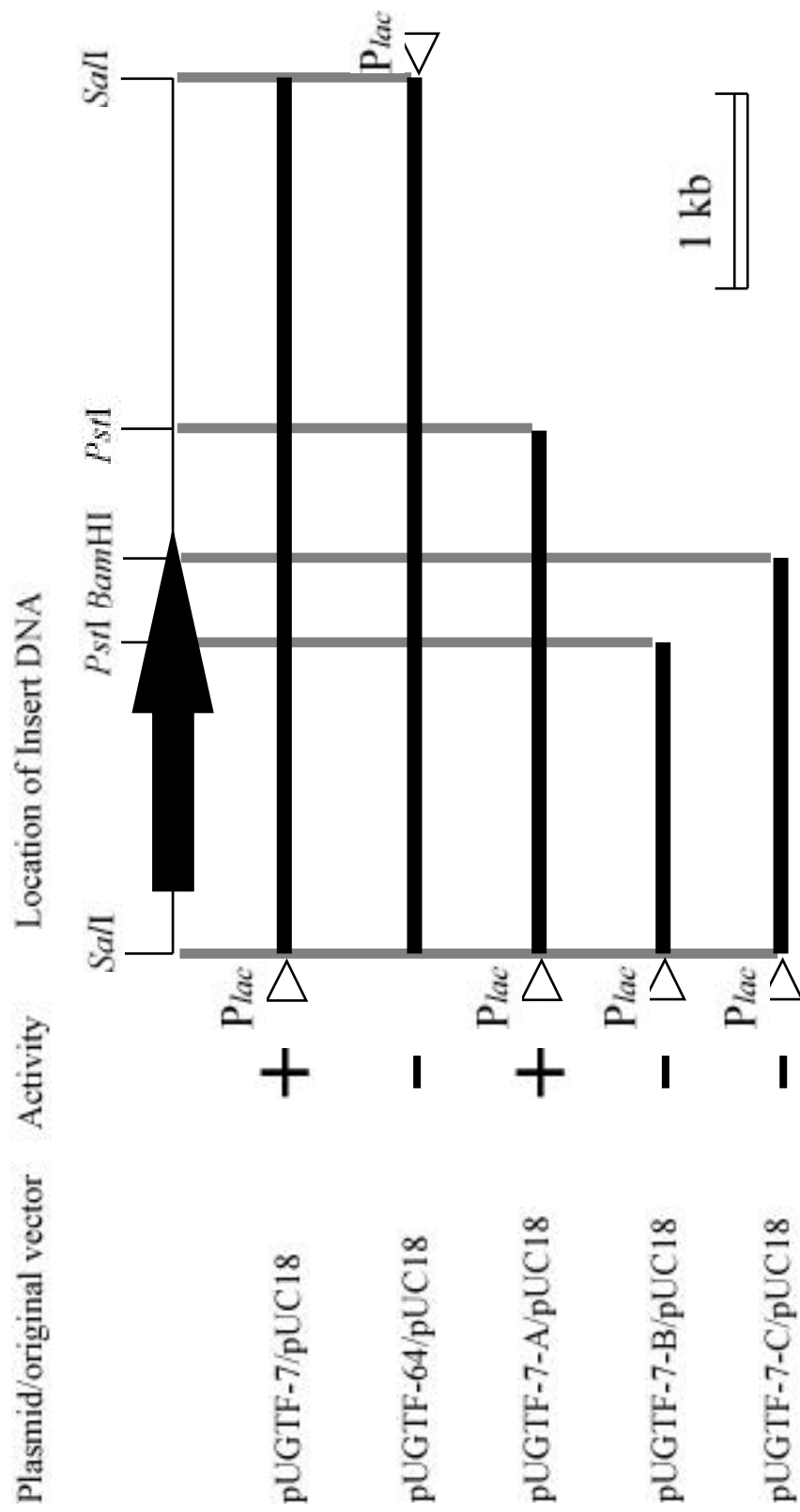
were determined , from the analysis of limited proteolytic digests of the purified enzyme. Based on these amino acid sequences, two oligonucleotide primers were synthesized as described in Materials and Methods. A DNA fragment of approximately 180-bp was amplified by PCR using total DNA of *X. campestris* WU-9701 as a template with the primers. Through the selection with Southern

hybridization and colony hybridization using DIG-labeled 180-bp PCR amplified product, two positive clones harboring the plasmids, designated as pUGTF-7 and pUGTF-64 respectively, containing the same 4.3-kb *SalI* fragment in pUC18 were isolated from 3000 individual colonies (Fig. 6.1). As shown in Fig. 6.2, an open reading frame (ORF) of *xgtA* was located under the same orientation with *lac* promotor of pUC 18 as for pUGTF-7. On the other hand, the gene was located under the reverse orientation with *lac* promotor as for pUGTF-64.

### 6.3.2 Nucleotide sequence and identification of *xgtA*

As for the cell-free extract of *E. coli* JM109/pUGTF-7 and *E. coli* JM109/pUGTF-64,  $\alpha$ -glucosyl transfer activity was detected only for *E. coli* JM109/pUGTF-7, but not *E. coli* JM109/pUGTF-64, even after cultivation in the presence of IPTG. These results indicated that the ORF of *xgtA* was expressed under the control of *lac* promotor in *E. coli* JM109/pUGTF-7, and that the promotor of *xgtA* was not functionable in *E. coli* at least under the cultivation conditions tested.

Several deletion plasmids were constructed through the restriction enzyme-digestion of 4.3 kb-*SalI* fragment in pUGTF-7. The plasmid containing 2.5-kb *SalI*-*PstI* fragment was designated as pUGTF-7-A, and was expressed in *E. coli* JM109. The plasmids lacking 230 bp and 470 bp of 3'-side of *xgtA* gene were constructed and designated as pUGTF-7-B and pUGTF-7-C, respectively, as shown in Fig. 6.2. However,  $\alpha$ -glucosyl transfer activity was not detected for



**Fig. 6.2.** Genetic organization and restriction map of the 4.3-kb *SalI* fragment including *xgtA*. Black arrow indicates *xgtA* encoding  $\alpha$ -glucosyl transfer enzyme. White arrows indicate the directions of *lac* promoter. Closed bars of pUGTF-7 and pUGTF-64 indicate the insert DNA in recombinant plasmids carrying *xgtA* in pUC18. Activities: +,  $\alpha$ -glucosyl transfer activity was detected; -, not detected.

either *E. coli* JM109/pUGTF-7-B or *E. coli* JM109/pUGTF-7-C. Moreover, maltose hydrolysis activity was also slightly detected for both *E. coli* JM109/pUGTF-7-B and *E. coli* JM109/pUGTF-7-C compared with that of *E. coli* JM109/pUGTF-7 (data not shown). From these results, 3'-side of *xgtA* gene was confirmed to be essential for  $\alpha$ -glucosyl transfer and maltose hydrolysis activities of XgtA.

As shown in Fig. 6.2, a restriction map was constructed, and a 2.5-kb *SalI*-*PstI*-digested fragment was identified as the one containing *xgtA* gene. Therefore, the nucleotide sequence of the *SalI*-*PstI* fragment was determined and the gene *xgtA*, corresponding to the  $\alpha$ -glucosyl transfer enzyme, was identified to be 1617 bp, which encodes 57 kDa protein consisting of 539 amino acids residues, as shown in Fig. 6.3. A putative ribosome-binding site was found 9-bp upstream of ATG codon.

The predicted amino acid sequence of *xgtA* exhibited homologies with those of several enzymes classifiable into the  $\alpha$ -amylase family: 56% for putative  $\alpha$ -glucosidase (EC 3.2.1.20) of *Sinorhizobium meliloti*<sup>1)</sup>, 35% for trehalose-6-phosphate hydrolase (EC 3.2.1.93) of *E. coli*, 33% for  $\alpha$ -glucosidase of *Saccharomyces cerevisiae*, 32%, 32%, and 31% for oligo-1, 6-glucosidases (EC 3.2.1.10) of *Bacillus cereus*, *B. coagulans*<sup>2)</sup> and *B. thermoglucosidasius*, respectively (Table 6.1). However, the author has already confirmed that XgtA shows no activity of oligo-1, 6-glucosidase and trehalose-6-phosphate hydrolase (details not shown). On the other hand, the predicted amino acid sequence of XgtA exhibited less than 20% homology with the enzymes of eucaryotes, except for  $\alpha$ -glucosidase of *S. cerevisiae*.



Table 6.1. Comparison of the gene *xgtA* with other enzyme genes.

Enzyme	Identity (%)	
	Amino acid	Nucleotide
-Glucosidase from <i>Sinorhizobium meliloti</i>	56.2	62.6
Trehalose-6-phosphate hydrolase from <i>Escherichia coli</i>	35.0	51.8
-Glucosidase from <i>Saccharomyces cerevisiae</i>	33.3	47.0
Oligo-1, 6-glucosidase from <i>Bacillus cereus</i>	31.6	47.9
Oligo-1, 6-glucosidase from <i>Bacillus coagulans</i>	31.5	47.2
Oligo-1, 6-glucosidase from <i>Bacillus thermoglucosidasius</i>	31.2	47.6
Dextran glucosidase from <i>Streptococcus mutans</i>	30.4	47.3

A comparison of the deduced amino acid sequences is shown in Fig. 6.4, indicating that several regions near the N-terminal region are completely conserved. The conserved regions (1-372 amino acids) in XgtA correspond to those encoding ( / )8 barrel structure specific for the enzymes belonging to the enzymes of -amylase family<sup>3)</sup>. The other conserved regions found in XgtA (99 to 104, 197 to 205, 253 to 256 and 326 to 331 amino acid residues,



XgtA	1	-----MSQT--PWWRGAVIYQIYPRSFIDNSGDGVGDLPLGIIITAKLDYISGLGVDAIWIWSPFEKSPMAIDFGYDTSIDYRAVDPLFGSLADFFDRLEKA	89
AgIA	1	MTMNETTSSLLLEADRDRWRRPVIIYQIYPRSFQDINGDGLQGITARLPHIAGLGADAIWISPEFTSPMRDFGYDSNYVDVDPFIIGTLDFDALIAEA	100
TreC	1	-----MTHLPHW--WQNGVIYQIYPKSFQDITGSGTGLGRGVTHLDYLHLGVDAIWLTPFYVSPQDNGYDVANYTAIPTYTGLDDFDELVTQA	90
MalL	1	-----MTEW--WKKAVVYQIYPRSFIDNGDGLGRGIMDKLDYLLKTLGIDICIIWISPYVYDSDPQDNGYDIRYRKTDKMGFTNEDMDRLIDEA	87
XgtA	90	HGLGLKVMIDQVLSHTSIAHAWFQESQDRSNPKADWYVMAIDPREDDGTPPNWMLSLFEGVAWQWEPREQYVYLNHFLVDQPDNLNFHNAEYQQATLNDNVR	189
AgIA	101	HRLGLRMVIDLVLSTSDRIHPWFVESRSRNSAKADWYVMAIDSKPDGTPPNWMLSLFEGSAWQWDPTRLQYVYLNHFLTSQPDNLHNPQVQEAALLAVER	200
TreC	91	KSRGIRIILDMVFNHTSTQHAWFREALNKE-SPYRQFYWRDGEPETP-PNNWRSKFSGSAWRHAESEQYVYHLFAPEQADLNWENPAVRAELKKVCEF	188
MalL	88	HARGIKIVMDLVNHTSDEHAWFVESRSKSDNPYRDFYFQDKPDGTPPNWNGSMFSGSAWEYDETTGQYVYHLYFSKKQPDNLWENENEKVRKEIYDMMKF	187
XgtA	190	WLDRGVDGFRLLDAINFC-----FHDAQIRDNPAPKADKRVGRGFSADNPYAYQYHYFNNTQPENLPFLRLRGLLDSYPGAVSLGEISSEDS-LATTAEY	283
AgIA	201	WLERGVDGFRLLDTINFY-----FHDREIRDNPALVPERRNASTAPAVNPYQEHYDKNRPENL-----EFLKRFRAVWDEFAPATAAVGEVGDQSQRGLEIA	292
TreC	189	WADRGVDGRLLDVNLISKDPRIPE-DIDGDGRR-----FYTD-GPRAHEFLHEMNRDVFTPRGLMTGVGEMSSSTSLHCQRYAALTGSELSMTFNFHL-K	281
MalL	188	WMDKGVDGWRMDVIGSISKFLDEPDYEPEGQ-KYGIGKYHANGPRLHAFIQEMNREVL SKYDCMTVGEATGSDVEIARKYTGPDRLHNLNMFNFEHM-D	285
XgtA	284	TAQGR-LHMGYSELLVQDYSAAY-----IRDTVSRLEATMTLEGWPCWAI SNHDVVRATRWGGAQTAF-ARMVVALLCSLRGSICLYOGEELGLSEA-	376
AgIA	293	GEYTSGGDKVHMCYAFELAPDRLTPQVAVELRDFHRAAPEGWACWAFSNHDVVRHVSRWADGVTDHDAHAKLLASLMSLRGTVCITYOGEELALAEA-	391
TreC	282	VDYPG-----GEKWTLLAKP-DFVALKTLFRHWQQGMHNV-AWNAALFWCHNDQPRIIVSRFRGDEGEYRPAAKMLAWLHGMQGTPIITYOGEELIGMTNPH	372
MalL	286	VDTKPGSPA-----G-KWALK-PFDLVELKQILSRWQYELADT-GWNAALYFENHDQARVWSRWGNDTTYRAECAKAFATLHGLKGTPTPTTYOGEELIGMVNAD	379
XgtA	377	-----EVAFE--DLQDPYGITWPTFKGRDGC-----RTPMPWTDAPSAGFTSGKPMPLAASH-RAAAVSQ-QDDAHSVLRAVRAFLAWRKE	456
AgIA	392	-----ELDYE--DLQDPMASSSGPTSSAGR-C-----RTPMVWESLDPDGGESATPMWIPISQSHR-GRCRAGGRP-GLGAHYRR-FLAARKA	469
TreC	373	FTRITDYRDVESLNMFAELRNDGRDAD--ELLATLASKSRDNRTPMQWNSGDNAGFTAGEPMIGLGDNYQOINVEAALADDSVFTYQKLLIAL--RKQ	468
MalL	380	LELE-EYDDIEIRNAYQELVMENQIMSKDEFLTAVRKKGRDNARTPMQWDGSGNAGFTTGTPMWIKVNSRYSEINAKALQEPDISIFYYYSQSLIKL--RHS	476
XgtA	457	MPALREGSTIAFYDTAEPVLM--FRREHAGQGVLLAFNLISADPAELALPAGWEFQIDVPQVELGAMDGGHRLRAGHAVVAAGRG	538
AgIA	470	NPALAKGEIEFVETRGSLLG--FLRSHGNEKVFCLFNMSEAAATKELPMKRLEPLEGHGFVSEILDHEV-KLPWAGAFFARLA	549
TreC	469	EAILTWGNYQDL-LPNSPVLWCYRRRWKQGTLLVIANLSREIQWQAGQMRGNWQLVMHNYEEASPPQCAMNLRHPFEAVW-WLQK	551
MalL	477	YDVFTDGRYE-LMPDHPHLYVYTYRENESEKLLVAANLSENTVSFDQDPD--NWKLLLGNYEDTGT--TLFRPYEAAITYYLEK	555

**Fig. 6.4. Multiple alignment of the amino acid sequence of  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701 with several enzymes classifiable into the  $\alpha$ -amylase family. The amino acid residues which are identical in all the members are indicated by white letters in black boxes. The amino acid residues which are identical in more than half of the members are indicated by shadow boxes. Numbers indicate the positions of the residues in the complete amino acid sequence of the enzyme. XgtA,  $\alpha$ -glucosyl transfer enzyme of *Xanthomonas campestris* WU-9701 (Accession No. AB081949); AgIA, putative  $\alpha$ -glucosidase of *Sinorhizobium meliloti* (Accession No. AF045609); TreC, Trehalose-6-phosphate hydrolase of *Escherichia coli* (Accession No. L06097); Mall, oligo-1,6-glucosidase of *Bacillus cereus* (Accession No. P21332).**

respectively) correspond to “Regions 1-4” termed for the specific regions for the  $\alpha$ -amylase family<sup>3,4</sup>. These results clearly indicate that the  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701 is classifiable into the enzyme of  $\alpha$ -amylase family and seems to be classifiable into the “Family 13”<sup>3,4</sup>. On the other hand, the C-terminal region of XgtA, especially 423-539 amino acid residues, is less conserved than the N-terminal region. It should also be noted that the nucleotide and deduced amino acid sequences of *xgtA* showed slight homology to those of the genes encoding  $\alpha$ -glucosidases (EC. 3.2.1.20) functionally characterized although XgtA shows 56% homology to the “putative  $\alpha$ -glucosidase” of *S. meliloti*<sup>1)</sup> which is only a predicted one from the nucleotide sequence. On this point, the author will discuss later.

Genomic Southern hybridization was carried out using the plasmid pUGTF-7-A as a probe and *Eco*RI-, *Hind*III-, and *Sal*I-digested total DNA of *X. campestris* WU-9701 as a templates, and the results indicated that there is only one copy of *xgtA* in *X. campestris* WU-9701 chromosomes (data not shown).

### **6.3.3 Putative secondary structure and putative stereo structure of XgtA**

For prediction of structure of XgtA against the structure deposited in the Protein Data Bank, LIBRA analysis was carried out. Consequently, a high score was found between XgtA and *Bacillus cereus* oligo-1, 6-glucosidase, which hydrolyzes non-reducing terminal  $\alpha$ -1, 6-glucosidic bonds of isomaltosaccharides, panose and an  $\alpha$ -limit dextrin<sup>5)</sup>.

Putative secondary structure was predicted by using SS-Thread software. As shown Fig. 6.5, the region of 367-539 in XgtA was different from that of oligo-1, 6-glucosidase of *B. cereus*, in which the corresponding region was considered to be a part of N-terminal domain. In N-terminal domain of oligo-1, 6-glucosidase, the catalytic residues (Asp199, Glu255 and Asp329) are existed.

Putative structure of XgtA was drawn based on that of oligo-1, 6-glucosidase of *B. cereus*, whose structure was already determined<sup>5)</sup>, using 3D-JIGSAW software. When the structure of XgtA was overlaid with the three-dimensional structure of oligo-1, 6-glucosidase of *B. cereus*, the region of 373-401 in XgtA was different from that of oligo-1, 6-glucosidase of *B. cereus*, corresponding to blue region as shown in Fig. 6.6. Although, Watanabe et al.<sup>5)</sup> reported that the region (386 to 412) of oligo-1, 6-glucosidase might be related to the stability for temperature, the function of the region (373-401) of XgtA has not yet been characterized.

## 6. 4 Discussion

In this chapter, the gene (*xgtA*) encoding an  $\alpha$ -glucosyl transfer enzyme, catalyzing  $\alpha$ -anomer-selective glucosylation, was cloned. A gene 4.3 kb-*SalI* fragment was found to contain the 1617 bp open reading frame of *xgtA*, which encodes 57 kDa protein consisting of 539 amino acid residues (Fig. 6.3). A database search revealed that XgtA shows homologies to several enzymes classifiable into the  $\alpha$ -amylase family, "Family 13" (Fig. 6.4). The database

Table 6.2. Compatible structures of the enzymes with XgtA using LIBRA.

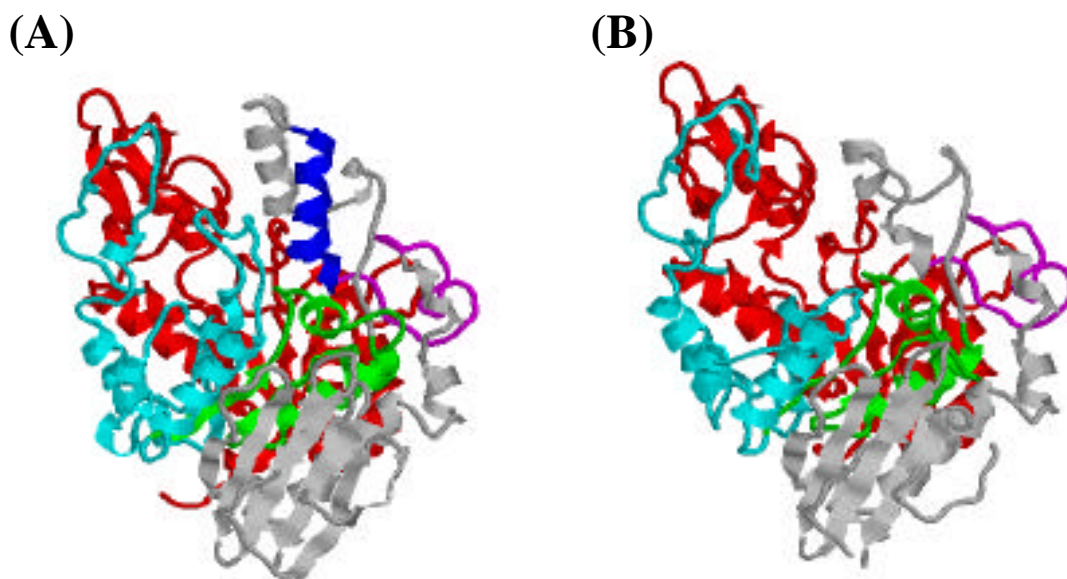
Enzyme and its source	Score
Oligo-1, 6-glucosidase from <i>Bacillus cereus</i>	-5.44
-Amylase II from <i>Thermoactinomyces vulgaris</i>	-4.69
Cyclodextrin glycosyltransferase from <i>Bacillus circulans</i>	-3.72
Glycosyltrehalose trehalohydrolase from <i>Sulfolobus solfataricus</i>	-3.46
-Amylase from Boar	-3.40

research revealed that XgtA shows 56% homology to the *S. meliloti* putative -glucosidase (AglA). Although the operon structure related to the gene *aglA* was reported<sup>1)</sup>, the gene *aglA* was determined to be a putative one based on the homology search by database and the gene product AglA in *S. meliloti* has not yet been characterized as an “-glucosidase” by enzyme assay. Recently, Silva et al.<sup>6)</sup> sequenced a genome of *X. campestris* pv. *campestris* to obtain an information of a pathogenic source and found a gene (*aglA*) as the one encoding a “putative -glucosidase”. The database research revealed that the deduced amino acid sequence of the gene *aglA* shows a 97% homology to that of XgtA. However, it should be noted that the gene *aglA* was also a putative one based on the homology search by database, and that the gene product AglA of *X. campestris* pv. *campestris* has not yet been characterized.

In addition, putative structure of XgtA was overlaid with the three-

XgtA 1' MSQTPWWRGA VLYQIYPRSF LDSNGDGVGD LPGIIAKLDY ISGLGVDAIW ISPFKSPMA  
 \* \* \* \* \*  
 Bce 1" MĚKQWWKĚŠ VVYQIYPRSF MDSNGDGIGD LRGLIŠKLDY LĚLĚGIDVIAW ISPVYĚSPND  
 \* \* \* \* \*  
 XgtA 61' DFGYDISDYR AVDPLFGSLA DFDRLLEKAH GLGLKVMIDQ VLSHTSIAHA WFQESRQDRS  
 \* \* \* \* \*  
 Bce 60" DNGYDISDYC KIMNEFGTME DWDFLIHEMH ERNMKIMMDL VVNHTSDEHN WFIERSKSKD  
 \* \* \* \* \*  
 XgtA 121' NPKADWYVWA DPREDGTPPN NWLSLFGGVA WQWEPRREQY YLHNFLVDQP DLNFHNAEVQ  
 \* \* \* \* \*  
 Bce 120" NKYRDYYIW- RPGKĚGKEPN NWGAAFSGSA WQYDEMTĚY YLHLFSKKQP DLNWDNĚKVĚ  
 \* \* \* \* \*  
 XgtA 181' QATLDNVREW LDRGVDGFR L DAINFCFHDA QLRDNPAKPA DKRVGRGFSA DNPYAYQYHY  
 \* \* \* \* \*  
 Bce 179" QDVYEMMKĚW LĚKGIDGERM DVINFIŠKĚĚ GLPTVETĚĚĚ ĚYVSGHKHFM NĚPNIHĚKY--  
 \* \* \* \* \*  
 XgtA 241' FNNTQPENLP FLERLR--GL LDSYPGAVSL GEISSEDSLA TTAETATQGR LHMGYSEFLL  
 \* \* \* \* \*  
 Bce 237" LĚHEMNEEVLS HYD IMTVGEM PGVTTEĚAKL YTĚĚĚRĚĚLQ MVEQF-ĚHMD LDSĚĚĚĚKWD  
 \* \* \* \* \*  
 XgtA 299' VQDYSAAYIR DTVSRLEATM -LEGWPCWAI SNHDVVRAVT RWG-GAQTAP AFARMVVAII  
 \* \* \* \* \*  
 Bce 296" VKPĚSLITLĚ ĚNI TKWĚKĚL EHTGWNŠLYW NNHDQPRVVS RFGNDĚMYR ĚSAKMIĚTVL  
 \* \* \* \* \*  
 XgtA 357' CSLRGSICLY QGEELGLSE- ---AEVAFED LQ--DPY--- -----GI TFWPTF-KGR  
 \* \* \* \* \*  
 Bce 356" HMMKGTPYIY QGEEIGMTNV RFĚŠIDĚYRD IĚTLNMYKEK VMERĚĚĚĚĚK VĚQŠIYIKGR  
 \* \* \* \* \*  
 XgtA 399' DGCRTMPWWT DAPSAGFTSG KPWLPLAASH RAAAVSVQQD DAHSVLRRAVR AFLAWRKEMP  
 \* \* \* \* \*  
 Bce 416" DNARTPMQWD DQNHAGFTTG ĚPWITVNPNY KĚINVKQĚIQ NĚKDSI ĚYFFYK KĚIĚLRKNNĚ  
 \* \* \* \* \*  
 XgtA 459' ALREGSIĚF- YDTĚPVLME RREHAGQVVL LAFNLSADPA ELALPAGEWE QIDVPGVELG  
 \* \* \* \* \*  
 Bce 476" IĚVYGSYDLI LĚNNPŠIĚĚY VRTYĚVĚKLL VIANFTĚĚĚC IĚĚLPĚDISY SEVELLIHNY  
 \* \* \* \* \*  
 XgtA 518' AMDGGHLRLA GHAVVAAVGR G  
 Bce 536" DVENGPIENI TLRPYEAMVF KLK

**Fig. 6.5. Putative secondary structure of XgtA and complete secondary structure of oligo-1, 6-glucosidase from *Bacillus cereus*. Secondary structure elements of XgtA was searched using SS-Thread. Red and blue underlined regions indicate  $\alpha$ -helix and  $\beta$ -strand, respectively.**



**Fig. 6.6. Stereoview of the predicted structure of  $\alpha$ -glucosyl transferase (XgtA), from *X. campestris* WU-9701, overlaid with the crystal structure of oligo-1, 6-glucosidase of *Bacillus cereus*. (A), oligo-1, 6-glucosidase of *B. cereus*; (B), XgtA. Molecular modeling of XgtA was performed using 3D-DIGSAW software.**

dimensional structure of oligo-1, 6-glucosidase of *B. cereus* using 3D-JIGSAW software. The structure of XgtA was overlaid with the three-dimensional structure of oligo-1, 6-glucosidase of *B. cereus*, the region of 373-401 in XgtA was different from oligo-1, 6-glucosidase of *B. cereus*, corresponding to the blue region as shown in Fig. 6.6. Watanabe et al.<sup>5)</sup> reported that the region (386 to 412) of oligo-1, 6-glucosidase was related to the stability for temperature. The corresponding region (373-401) in *X. campestris* WU-9701 has not yet been characterized, but two  $\alpha$ -helix structures in oligo-1, 6-glucosidase of *B. cereus* (grey and blue regions) seem to be disappeared in the corresponding regions in XgtA. These structural difference might be related to the specific enzymatic

functions of XgtA, as shown in Chap. 5.

## 6.5 References

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M. C. Alves, A. M. do Amaral, M. C. Bertolini, L. E. A. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L. P. Ciapina, R. M. B. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. S. Ferreira, R. C. C. Ferreira, M. I. T. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, E. G. M. Lemos, M. V. F. Lemos, E. C. Locali, M. A. Machado, A. M. B. N. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. M. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. M. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, A. Rossi, J. A. D. Sena, C. Silva, R. F. de Souza, L. A. F. Spinola, M. A. Takita. R. E. Tamura, E. C. Teixeira, R. I. D. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima.: Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature*. 417, 459-463 (2002).



## Chapter 7 Expression of the Gene Encoding a Novel -Glucosyl Transfer Enzyme of *Xanthomonas* *campestris* WU-9701 and Its Application to -Anomer-Selective Glucosylation of Menthol

### 7.1 Introduction

In Chap. 6, the gene *xgtA* of *Xanthomonas campestris* WU-9701 encoding a novel -glucosyl transfer enzyme, catalyzing -anomer-selective glucosylation of compounds having -OH groups was cloned. Since the amount of XgtA produced by *X. campestris* WU-9701 was limited for practical reaction system, high-level expression of *xgtA* is necessary for construction of an efficient -glucoside production system using XgtA.

In this chapter, the author describes that high-level expression of the gene *xgtA* in *Escherichia coli*, and that the recombinant enzyme XgtA produced by *E. coli* was utilized for -anomer-selective glucosylation of *l*-menthol and hydroquinone.

### 7.2 Materials and Methods

#### 7.2.1 Strains and plasmids

*E. coli* JM109 was used as a host for an expression of *xgtA*, and its genetic

type was described in Chap. 2. Plasmid pKK223-3 (Amersham Biosciences, NJ, USA) was used as a vector for expression.

### **7.2.2 Expression of the $\alpha$ -glucosyl transferase gene (*xgtA*) in *E. coli* JM109.**

For expression of *xgtA* in *E. coli* as a host, the recombinant plasmid pKKGTF was constructed. As described later, a DNA fragment of 1.6-kb, corresponding to the full length of *xgtA* from *X. campestris* WU-9701, was amplified by PCR with the oligonucleotide primers 5'-AGGGGAATTCATGTCGCAGACACCATG-3' and 5'-TGCAAGCTTTCAGCCACGACCGACAG-3'; the *Eco*RI- and *Hind*III-cleavage sites are underlined. The PCR product was digested with *Eco*RI and *Hind*III, and the *Eco*RI- and *Hind*III-DNA fragment of 1.6-kb was subcloned into the multicloning site of the vector pKK223-3. The resulting recombinant plasmid pKKGTF was used for the transformation of the host strain *E. coli* JM109.

### **7.2.3 Enzyme assay**

Cells of recombinant *E. coli* such as JM109/pKKGTF were grown under aerobic conditions with shaking at 37°C for 22 h in one liter of LB medium containing 100 mg/ml of ampicillin and 0.8 mM IPTG. The pH was initially adjusted to 7.0 with 2.0 M NaOH. After 22 h cultivation, cells were harvested

by centrifugation (6,000 × g, 15 min, 4°C), and washed twice with 10 mM citrate-10mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0). The cells were resuspended in 5 ml of 10 mM citrate-10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0), disrupted by sonication (20 kHz, 200 W, 10 min), and centrifuged (20,000 × g, 30 min, 0°C). The resulting supernatant 5 ml was collected as the cell-free extract.

-Glucosyl transfer activity was measured using maltose and hydroquinone as substrates as described in Chap. 2. A portion of 0.4 ml of the cell-free extract and 45 mM hydroquinone were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer containing 1.5 M maltose (pH 8.5) to give finally 1.2 M maltose in 2 ml of the reaction mixture, and incubated by shaking at 180 rpm at 40°C for 60 min. The reaction was stopped by heating in the boiling water at 100°C for 10 min. The amount of hydroquinone -glucoside formed was measured using high-performance liquid chromatography (HPLC), as described in Chap. 2. One unit (U) of -glucosyl transfer activity was defined as the amount of enzyme that produces one μ mole of hydroquinone -glucoside per minute from hydroquinone under the conditions described above. When maltose hydrolysis activity was measured, the same conditions for -glucosyl transfer activity described above were used with the exception that hydroquinone was omitted.

#### **7.2.4 -MenG synthesis by the recombinant enzyme of *E. coli* JM109/pKKGTF**

Unless otherwise indicated, glucosylation of *l*-menthol was carried out

under the standard reaction conditions as follows. Portions of 20 mg *l*-menthol and 0.4 ml of the cell-free extract of *E. coli* JM109/pKKGTF (1.2 U) were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 8.5) containing 1.5 M maltose to give finally 1.2 M maltose in 2 ml of the reaction mixture, and shaken at 180 rpm at 40°C for 3 h. The reaction was stopped by heating at 100°C for 10 min. Then, the reaction mixture was diluted with methanol up to 10 ml and filtrated on 0.20 μm PTFE membrane (Iwaki Glass Co., Ltd., Tokyo, Japan). The amounts of  $\alpha$ -MenG, glucose, and maltose were measured by HPLC with a Shimadzu LC-6A system with RID-6A differential refractometer (Shimadzu), as described in Chap. 2.

#### **7.2.5 Enzymatic synthesis of $\alpha$ -arbutin by $\alpha$ -anomer-selective glucosylation of hydroquinone using the recombinant enzyme of *E. coli* JM109/pKKGTF**

As described in Chap. 4, hydroquinone  $\alpha$ -glucoside is called as  $\alpha$ -arbutin and used as the material for cosmetics. Unless otherwise indicated, glucosylation of hydroquinone was carried out under the standard reaction conditions as follows. Portions of 20 mg hydroquinone and 400 μl of the cell-free extract of *E. coli* JM109/pKKGTF (1.2 U) were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 7.5) containing 1.5 M maltose to give finally 1.2 M maltose in 2 ml of the reaction mixture, and shaken at 180 rpm at 40°C for 3 h. The reaction was stopped by heating at 100°C for 10 min. Then, the reaction mixture was diluted with methanol up to 10 ml and filtrated on 0.20 μm PTFE

membrane (Iwaki Glass Co., Ltd., Tokyo, Japan). The amounts of hydroquinone -glucoside, glucose, and maltose were measured by HPLC with a Shimadzu LC-6A system with RID-6A differential refractometer (Shimadzu), as described in Chap. 2.

### **7.2.6 Other analytical methods**

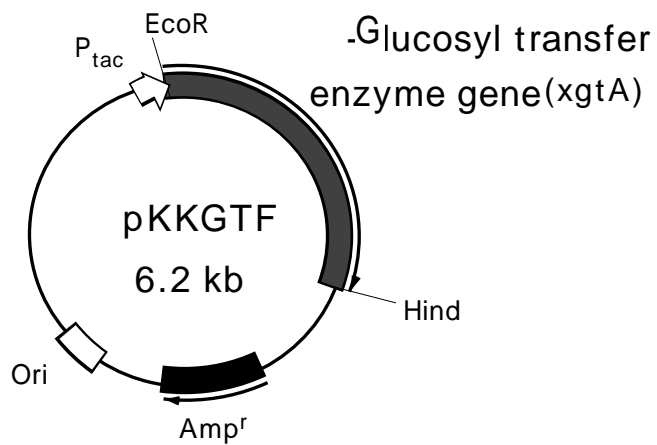
Protein concentration was determined by the method of Bradford<sup>1)</sup> using the Coomassie Protein Assay Kit (Pierce Chem. Co., Rockford, USA) with bovine serum albumin as a standard. For column chromatography, the protein concentration was measured by the absorbance at 280 nm using a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed using 10% (w/v) polyacrylamide by the method of Laemmli<sup>2)</sup>.

## **7.3 Results**

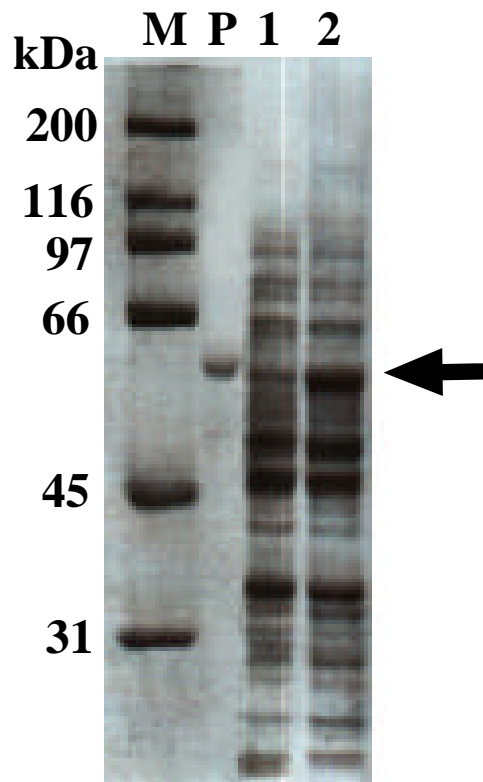
### **7.3.1 Expression of *xgtA* in *E. coli* and enzyme assay**

The expression plasmid containing *xgtA* gene was constructed and designated as pKKGTF as shown in Fig. 7.1. Cell-free extract of *E. coli* JM109/pKKGTF was prepared, and SDS-PAGE of the cell-free extracts of *E. coli* JM109/pKKGTF, as shown in Fig. 7.2, revealed that *E. coli*





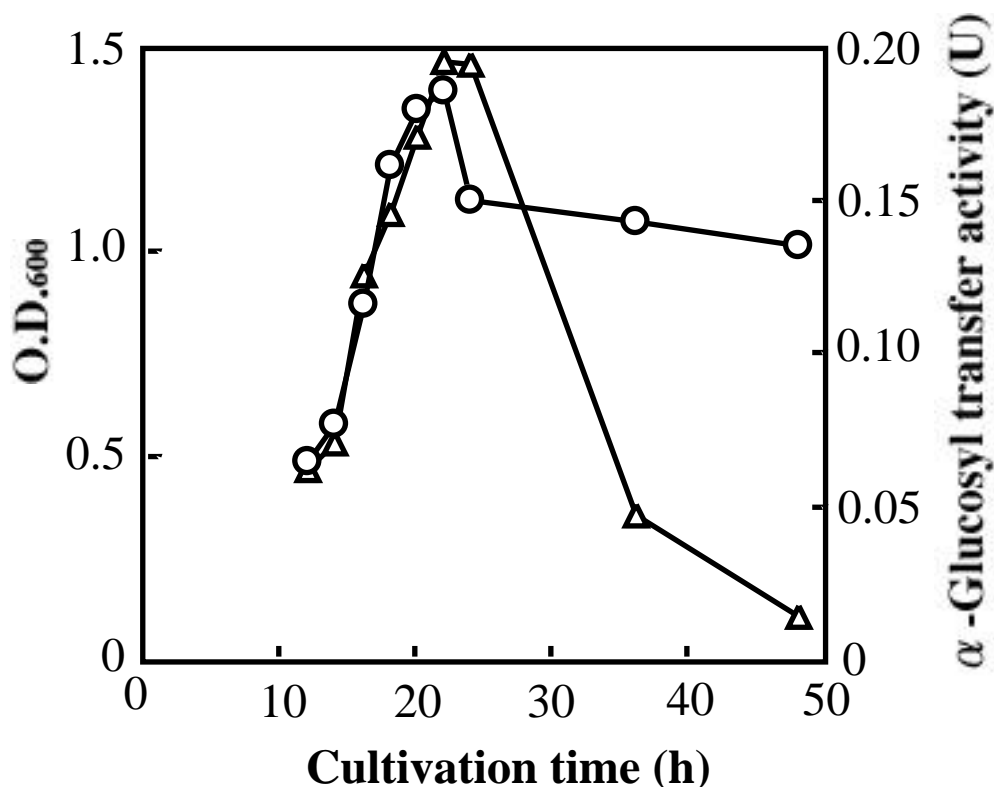
**Fig. 7.1. Physical map of pKKGTF.**



**Fig. 7.2 SDS-poly acrylamide gel electrophoresis of the cell-free extract of *E. coli* JM109/pKKGTF.**

**Lanes: M, molecular weight standards; P, native enzyme; 1, *E. coli*/pUGTF-7; 2, *E. coli*/pKKGTF.**

**The arrow indicates 57 kDa for the molecular weight of -glucosyl transfer enzyme.**



**Fig. 7.3.** Time course of O.D.<sub>600</sub> and α-glucosyl transfer activity of *E. coli* JM109/pKKGTF.

One unit (U) of α-glucosyl transfer activity was defined as the amount of enzyme that transfers one μ mole of α-glucose to hydroquinone from maltose per minute. Cells of *E. coli* JM109/pKKGTF were grown under aerobic conditions with shaking at 37°C for 22 h in one liter of LB medium containing 100 mg/ml of ampicillin and 0.8 mM IPTG. The crude enzyme was prepared as described in Materials and Methods. The amount of hydroquinone α-glucoside was measured by HPLC under the conditions as described in Chap. 2. Symbols: ○, O.D.<sub>600</sub>; △, α-glucosyl transfer activity.

JM109/pKKGTF produced mainly a protein of approximately 57 kDa, which is in accordance with the molecular weight of the α-glucosyl transfer enzyme

Table 7.1.  $\alpha$ -Glucosyl transfer activity of *E. coli* JM109/pKKGTF.

Origin	Specific activity (U/mg) <sup>a)</sup>	Relative
<i>X. campestris</i> WU-9701 <sup>b)</sup>	$3.5 \times 10^{-1}$	1
<i>E. coli</i> JM109/pKKGTF <sup>c)</sup>	4.8	140

<sup>a)</sup>One unit (U) of  $\alpha$ -glucosyl transfer activity was defined as the amount of enzyme that transfers one  $\mu$  mole of  $\alpha$ -glucose to hydroquinone from maltose per minute.

<sup>b)</sup>Cells of *X. campestris* WU-9701 were grown under aerobic conditions with shaking at 30°C for 48 h in one liter of medium containing (per liter) 50 g maltose, 2.0 g yeast extract (Difco, USA), 10 g peptone, and 1.0 g MgSO<sub>4</sub> · 7H<sub>2</sub>O. The pH was initially adjusted to 7.0 with 2.0 M NaOH. The crude enzyme was prepared as described in Chap. 3.

<sup>c)</sup>Cells of *E. coli* JM109/pKKGTF were grown under aerobic conditions with shaking at 37°C for 22 h in one liter of LB medium containing 100 mg/ml of ampicillin and 0.8 mM IPTG. The crude enzyme was prepared as described in Materials and Methods.

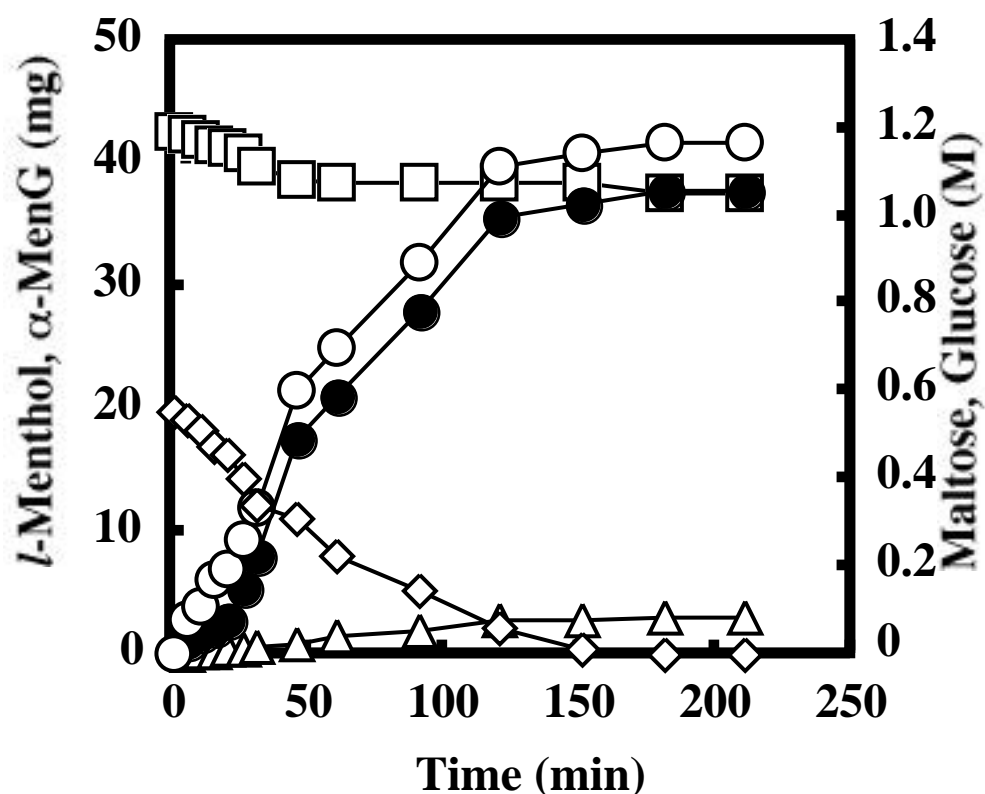
purified from *X. campestris* WU-9701, as described in Chap. 5. Time course of O.D.<sub>600</sub> and  $\alpha$ -glucosyl transfer activity of *E. coli* JM109/pKKGTF were investigated as shown in Fig. 7.3. Until 22 h, O.D.<sub>600</sub> and  $\alpha$ -glucosyl transfer activity of *E. coli* JM109/pKKGTF were increased. However, after 22 h, they were decreased since *E. coli* JM109/pKKGTF was bacteriolysed. The assay of  $\alpha$ -glucosyl transfer enzyme was done for the cell-free extract. As shown in Table 7.1, the specific activity of  $\alpha$ -glucosyl transfer enzyme reached  $1.4 \times 10^2$ -



fold over that of WU-9701. These results indicated that the recombinant XgtA produced in *E. coli* JM109/pKKGTF was fully active.

### **7.3.2 Application of the recombinant enzyme to $\alpha$ -anomer-selective glucosylation of *l*-menthol**

Since *E. coli* JM109/pKKGTF highly expressed *xgtA*, the cell-free extract of *E. coli* JM109/pKKGTF was prepared and used as the crude enzyme solution of recombinant XgtA for production of  $\alpha$ -MenG. Time course of  $\alpha$ -MenG production using recombinant XgtA, with 1.2 U/ml of reaction mixture under the optimum conditions (pH 8.5, maltose concentration 1.2 M, 40°C), is shown in Fig. 7.4. Within 1 h-reaction,  $\alpha$ -MenG was already accumulated mainly as a crystalline form in the reaction mixture since the amount of  $\alpha$ -MenG produced exceeded its saturated concentration. At 3 h, the total amount of  $\alpha$ -MenG reached a maximum of 42 mg, which corresponded to 99% molar conversion yield based on supplied *l*-menthol. In the reaction mixture, no maltooligosaccharide such as maltotriose and maltotetraose was produced as similar to the previous study using *X. campestris* WU-9701 enzyme<sup>3)</sup>. In a previous study using *X. campestris* WU-9701 enzyme<sup>3)</sup>,  $\alpha$ -MenG was produced with 99% molar conversion yield through 48 h-reaction under the similar conditions described above, except for the use of lyophilized cells of *X. campestris* WU-9701 in which  $5.5 \times 10^{-2}$  U/ml of the reaction mixture as a biocatalyst instead of the recombinant XgtA, was reported. In comparison with the previous study using *X. campestris* WU-9701 enzyme, the reaction time for



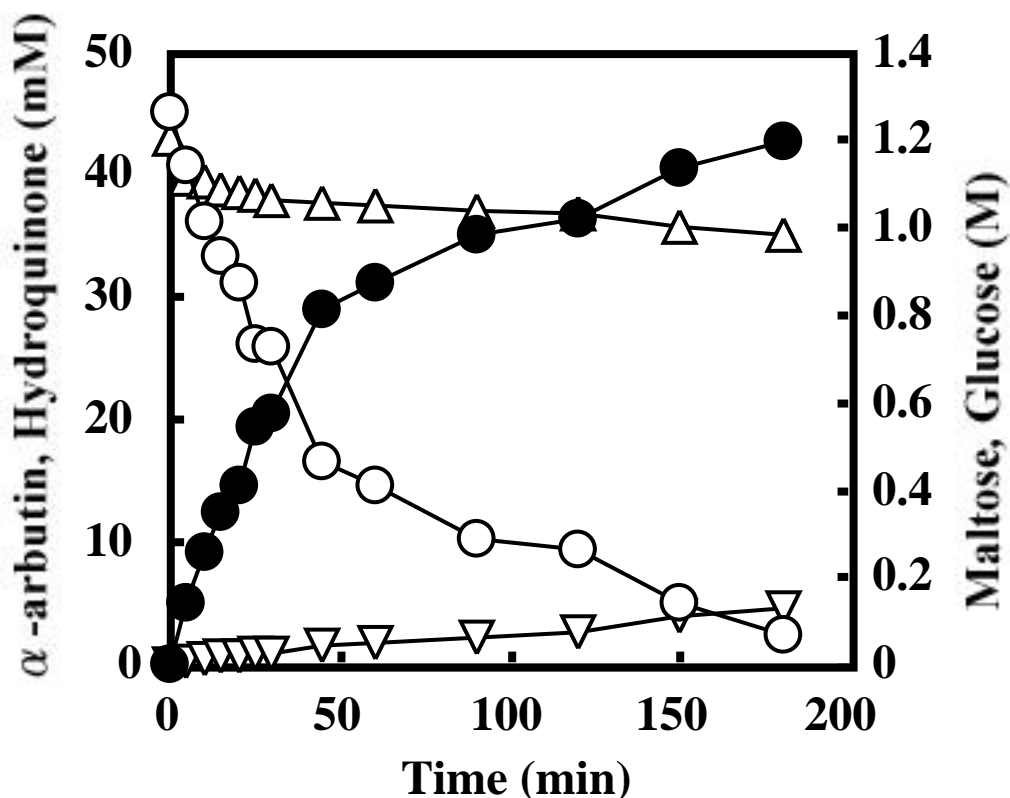
**Fig. 7.4.** Time course of the synthesis of  $\alpha$ -MenG using the recombinant enzyme of *E. coli* JM109/pKKGTF. The reactions were carried out under the optimal conditions: Portions of 20 mg *l*-menthol and 400  $\mu$ l of cell-free extract of *E. coli* JM109/pKKGTF (1.2 U) were added to 1.6 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 8.5) containing 1.5 M maltose to give finally 1.2 M maltose in 2 ml of the reaction mixture. The reaction mixture was shaken at 180 rpm at 40°C. Symbols:  $\circ$ , total  $\alpha$ -MenG;  $\bullet$ , crystalline  $\alpha$ -MenG (as precipitate);  $\square$ , *l*-menthol;  $\triangle$ , maltose;  $\blacktriangle$ , glucose.

$\alpha$ -MenG production to give the same molar conversion yield (99%) was drastically shortened from 48 h for the previous study<sup>3)</sup> using *X. campestris* WU-9701 enzyme to 3 h for the present study (Fig. 7.4). Therefore, the author

succeeded in the construction of an efficient  $\alpha$ -MenG production system using the recombinant XgtA.

### **7.3.3 Application of the recombinant enzyme to enzymatic synthesis of $\alpha$ -arbutin by $\alpha$ -anomer-selective glucosylation of hydroquinone**

The cell-free extract of *E. coli* JM109/pKKGTF was prepared and used for production of  $\alpha$ -arbutin. Time course of  $\alpha$ -arbutin production using recombinant XgtA, with 1.2 U/ml of reaction mixture under the optimum conditions (pH 7.5, maltose concentration 1.2 M, 40°C), is shown in Fig. 7.5. At 3 h, the total amount of  $\alpha$ -arbutin reached a maximum of 35 mg, which corresponded to 93% molar conversion yield based on supplied hydroquinone. In the reaction mixture, no maltooligosaccharide such as maltotriose and maltotetraose was produced as similar to the previous study using *X. campestris* WU-9701 enzyme. In the previous study, using WU-9701 enzyme,  $\alpha$ -arbutin was produced with 93% molar conversion yield through 36 h-reaction under the similar conditions described above, except for the use of lyophilized cells of *X. campestris* WU-9701 in which  $3.3 \times 10^{-1}$  U/ml of the reaction mixture as a biocatalyst instead of the recombinant XgtA, was reported in Chap. 4. In comparison with the results in Chap. 4 using *X. campestris* WU-9701 enzyme, the reaction time for  $\alpha$ -arbutin production to give the same molar conversion yield (93%) was drastically shortened from 36 h for the previous study using *X. campestris* WU-9701 enzyme to 3 h for the present study (Fig. 7.5). Therefore,



**Fig. 7.5.** Time course of the synthesis of  $\alpha$ -arbutin using the recombinant enzyme of *E. coli* JM109/pKKGTF. The reactions were carried out under the optimal conditions: Portions of 10 mg hydroquinone and 400  $\mu$ l of cell-free extract of *E. coli* JM109/pKKGTF (1.2 U) were added to 1.6 ml of 10 mM  $\text{H}_3\text{BO}_3$ -NaOH-KCl buffer (pH 7.5) containing 1.5 M maltose to give finally 1.2 M maltose in 2 ml of the reaction mixture. The reaction mixture was shaken at 180 rpm at 40°C. Symbols:  $\bullet$ ,  $\alpha$ -arbutin;  $\circ$ , hydroquinone;  $\blacktriangle$ , maltose;  $\triangle$ , glucose.

the author succeeded in the development of an efficient  $\alpha$ -arbutin production system using the recombinant XgtA.

## 7.4 Discussion

As described in the previous study<sup>3)</sup>, the enzymatic synthesis of  $\alpha$ -MenG by  $\alpha$ -anomer-selective glucosylation using the lyophilized cells of *X. campestris* WU-9701 with 99% of a molar conversion yield was already succeeded, but the reaction needed 48 h. In this study, for construction of an efficiency  $\alpha$ -glucoside production system, the author performed a high-level expression of *xgtA* in *E. coli*. The expression plasmid containing *xgtA* gene was constructed as pKKGTF as shown in Fig. 7.1. SDS-PAGE of the cell-free extracts of *E. coli* JM109/pKKGTF, as shown in Fig. 7.2, revealed that *E. coli* JM109/pKKGTF produced mainly a protein of approximately 57 kDa, which is in accordance with the molecular weight of the  $\alpha$ -glucosyl transfer enzyme purified from *X. campestris* WU-9701, as described in Chap. 5. Time course of O.D.<sub>600</sub> and  $\alpha$ -glucosyl transfer activity of *E. coli* JM109/pKKGTF were investigated as shown in Fig. 7.3. The value of O.D.<sub>600</sub> and  $\alpha$ -glucosyl transfer activity of *E. coli* JM109/pKKGTF were increased until 22 h with cultivation time, and thereafter, they were decreased by bacteriolysis of *E. coli* JM109/pKKGTF. Consequently, a high-level expression of *xgtA* in *E. coli* was succeeded, and the specific activity of  $\alpha$ -glucosyl transfer enzyme reached  $1.4 \times 10^2$ -fold as much as that of WU-9701 as shown in Table 7.1. These results indicated that the recombinant XgtA produced in *E. coli* JM109/pKKGTF was active and applicable to production of  $\alpha$ -glucoside.

As shown in Fig. 7.4, the author succeeded in  $\alpha$ -MenG production using recombinant XgtA from *l*-menthol and maltose. By a high-level expression of

*xgtA* in *E. coli* JM109/pKKGTF, 0.6 U/ml of XgtA was added to the reaction mixture. Even in the early reaction time (30 min),  $\alpha$ -MenG was mainly accumulated as a crystalline form in the reaction mixture. After 3 h, liquid *l*-menthol was completely consumed in the reaction mixture. These results were quantitatively confirmed also by HPLC(data not shown). At 3 h, the total amount of  $\alpha$ -MenG reached a maximum of 42 mg, which corresponded to 99.8% molar conversion yield based on supplied *l*-menthol. Even after 4 h, the amount of  $\alpha$ -MenG was maintained without being hydrolyzed (data not shown). Besides  $\alpha$ -MenG, no other by-products such as menthol derivatives or maltooligosaccharide were detected, and only glucose was accumulated in the reaction mixture. On the other hand,  $\alpha$ -arbutin synthesis using the recombinant XgtA with shortened reaction times from 36 h (results in Chap. 4 using *X. campestris* WU-9701 enzyme) to 3 h (the present study using the recombinant XgtA) was also succeeded as shown in Fig. 7.5. Even after 4 h, the amount of  $\alpha$ -arbutin was maintained without being hydrolyzed (data not shown). Besides  $\alpha$ -arbutin, no other by-products such as hydroquinone derivatives or maltooligosaccharide were detected, and only glucose was accumulated in the reaction mixture.

In conclusion, the author succeeded in the development of an efficient  $\alpha$ -glucoside production system using recombinant XgtA produced by *E. coli* expressing highly *xgtA*. Moreover, the author have confirmed that the  $\alpha$ -anomer-selective glucosylation of *l*-menthol and hydroquinone is catalyzed by the recombinant XgtA produced by *E. coli* 109/pKKGTF. Through the series of this thesis,  $\alpha$ -glucosyl transfer enzyme (XgtA) is not a kind of  $\beta$ -

glucosidase from enzymatic properties, kinetic properties and the predicted structure of XgtA.

## 7.5 References

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## Chapter 8 Summary and Conclusion

Some of organic compounds with useful biological activities have -OH groups. Glucosylation is considered to be one of the important methods for the structural modification of compounds having -OH groups since it increases water solubility and improves pharmacological properties of the original compounds. Enzymatic synthesis is superior as a method to the chemical synthesis in such cases that the enzymatic reactions proceed regioselectively and stereoselectively without protection and deprotection processes. In addition, the enzymatic reactions occur usually under mild conditions: at ordinary temperature and pressure, and a pH value around neutrality. For example, in the case of glucosylation of compounds to use for food additives, it is important to develop an one-step enzymatic synthesis method for  $\beta$ -glucosides since  $\alpha$ -glucoside compounds generally have a bitter taste.

*Xanthomonas campestris* WU-9701 produces a novel enzyme catalyzing  $\beta$ -anomer-selective glucosylation. This enzyme was able to utilize for one-step enzymatic synthesis of various  $\beta$ -glucosides since it catalyzed  $\beta$ -anomer-selective glucosylation of compounds having -OH groups using maltose as a glucosyl donor. In the reactions, no maltooligosaccharide such as maltotriose or maltotetraose was formed although byproduct oligosaccharides are generally produced by the usual  $\beta$ -glucosidases catalyzing mainly hydrolysis. From these results, the author considered that this enzyme was different from the typical  $\beta$ -glucosidase and decided to clarify the properties of the purified enzyme.



In this thesis, the author describes that he purified the  $\alpha$ -glucosyl transfer enzyme to give the single band on SDS-PAGE and characterized. Moreover, the author describes that he cloned the gene (*xgtA*) encoding the  $\alpha$ -glucosyl transfer enzyme, catalyzing  $\alpha$ -anomer-selective glucosylation, for high-level expression in *Escherichia coli*. The recombinant enzyme XgtA produced by *E. coli* was utilized for efficient production of valuable  $\alpha$ -glucosides by  $\alpha$ -anomer-selective glucosylation reaction.

In chapter 1, the methods for enzymatic synthesis of several glucosides and mechanism of xanthan gum synthesis by *Xanthomonas campestris* are described.

In chapter 2, some characteristics of the *Xanthomonas campestris* WU-9701, growth conditions of microorganisms, genetic manipulations, and experimental methods used for this thesis are described.

In chapter 3,  $\alpha$ -Anomer-selective glucosylation of (+)-catechin using the crude enzyme, showing  $\alpha$ -glucosyl transfer activity, of *Xanthomonas campestris* WU-9701 is described. When 60 mg of (+)-catechin and the enzyme ( $6.5 \times 10^{-1}$  unit as  $\alpha$ -glucosyl transfer activity) were incubated in 10 ml of 10 mM citrate- $\text{Na}_2\text{HPO}_4$  buffer (pH 6.5) containing 1.2 M maltose at 45 °C, only one (+)-catechin glucoside was selectively obtained as a product. The (+)-catechin glucoside was identified as (+)-catechin 3'-*O*- $\alpha$ -D-glucopyranoside ( $\alpha$ -C-G) by  $^{13}\text{C}$ -NMR,  $^1\text{H}$ -NMR and two-dimensional HMBC analysis. The reaction at 45 °C for 36 h under the optimum conditions gave 12 mM  $\alpha$ -C-G, 5.4 mg/ml in the reaction mixture, and the maximum molar conversion yield based on the amount of (+)-catechin supplied reached 57%. At 20 °C, the

solubility in pure water of  $\beta$ -C-G, of 450 mg/ml, was approximately 100-fold higher than that of (+)-catechin, of 4.6 mg/ml. Since  $\beta$ -C-G has no bitter taste and a slight sweet taste compared with (+)-catechin which has a very bitter taste,  $\beta$ -C-G might be a desirable additive for foods, particularly sweet foods.

In chapter 4, enzymatic synthesis of  $\beta$ -arbutin by  $\beta$ -anomer-selective glucosylation of hydroquinone using the lyophilized cells of *Xanthomonas campestris* WU-9701 is described.  $\beta$ -Arbutin, a useful cosmetic ingredient, was selectively synthesized by  $\beta$ -anomer-selective glucosylation of hydroquinone with maltose as a glucosyl donor using lyophilized cells of *Xanthomonas campestris* WU-9701 as a biocatalyst. When 45 mM hydroquinone and lyophilized cells of WU-9701 showing  $6.6 \times 10^{-1}$  unit of  $\beta$ -glucosyl transfer activity were shaken in 2 ml of 10 mM H<sub>3</sub>BO<sub>3</sub>-NaOH-KCl buffer (pH 7.5) containing 1.2 M maltose at 40 °C, only one form of hydroquinone glucoside was selectively obtained as a product and identified as hydroquinone 1-*O*- $\beta$ -D-glucopyranoside ( $\beta$ -arbutin) by <sup>13</sup>C-NMR, <sup>1</sup>H-NMR and two-dimensional HMBC analysis. Although hydroquinone has two phenolic -OH groups in the *para* positions in its structure, only one -OH group, but not both -OHs, was glucosylated and no other glucosylated products such as maltotriose were detected in the reaction mixture. The reaction at 40 °C for 36 h under optimum conditions yielded 42 mM  $\beta$ -arbutin, and the maximum molar conversion yield based on the amount of hydroquinone supplied reached 93%.

In chapter 5, the purification and characterization of  $\beta$ -glucosyl transfer enzyme, catalyzing  $\beta$ -anomer-selective glucosylation, produced by *X. campestris* WU-9701 are described. This enzyme was 99.6-fold purified from a

cell-free extract of WU-9701 after sonication through ammonium sulfate precipitation, DEAE-Toyopearl 650S anion exchange chromatography, gel filtration, maltose-agarose chromatography and two steps-Q-Sepharose anion exchange chromatography. The molecular weights of the enzyme were estimated to be 57 kDa by SDS-polyacrylamide gel electrophoresis and 60 kDa by Superdex 200 gel filtration, indicating that the enzyme is monomeric one. The enzyme was inhibited by  $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Zn^{2+}$  and *p*CMB and activated by  $K^+$  and  $Na^+$ . Without  $\alpha$ -glucosyl acceptors such as menthol and hydroquinone, the enzyme hydrolyzed a slight amount of maltose but not maltotriose or sucrose. With  $\alpha$ -glucosyl acceptors, the enzyme showed  $\alpha$ -glucosyl transfer activity to produce readily the corresponding  $\alpha$ -glucosides. However, toward mono- and saccharides such as glucose, fructose, galactose, sucrose and  $\beta$ -glucosyl transfer reaction did not occur. These results clearly indicated that the enzyme is not an usual  $\alpha$ -glucosidase mainly catalyzing hydrolysis reaction. Therefore, it was concluded that the enzyme of *X. campestris* WU-9701 is a unique one catalyzing mainly  $\alpha$ -glucosyl transfer reaction, and referred to as a novel " $\alpha$ -glucosyl transfer enzyme".

In chapter 6, cloning and sequencing of a gene (*xgtA*) encoding the  $\alpha$ -glucosyl transfer enzyme of *X. campestris* WU-9701 are described. A gene 4.3 kb-*SalI* fragment contained the 1617 bp open reading frame of *xgtA*, which encodes 57 kDa protein consisting of 539 amino acid residues. The deduced primary amino acid sequence of XgtA shows homologies to those of several enzymes such as putative  $\alpha$ -glucosidase of *Sinorhizobium meliloti* (56%) and oligo-1, 6-glucosidases of *Bacillus cereus*, *B. coagulans* and *B.*

*thermoglucoasidarius* (31-32%), but has slight identity with those of other enzymes containing known  $\alpha$ -glucosidases. Moreover, the region corresponding to C-terminus of XgtA, especially 423 to 539 amino acids residues, shows slight homology to any other enzymes. In addition, the 3D structure of XgtA predicted from the primary structure of XgtA was drawn based on that of oligo-1, 6-glucosidase of *B. cereus*.

In chapter 7, high-level expression of *xgtA* in *Escherichia coli* and utilization of the recombinant enzyme produced by *E. coli* for  $\alpha$ -anomer-selective glucosylation of *l*-menthol are described. The ORF of *xgtA* was subcloned in pKK223-3, and the chimeric plasmid pKKGTF was constructed and expressed in *E. coli* JM109 under the control of *tac* promoter of pKK223-3. The specific activity of *E. coli* JM109/pKKGTF reached 4.8 unit/mg, being  $1.4 \times 10^2$ -fold as much as that of WU-9701. The reaction mixture containing maltose and the cell-free extract of *E. coli* JM109/pKKGTF expressing *xgtA* was utilized for  $\alpha$ -anomer-selective glucosylation of *l*-menthol. Through 3 h-reaction, *l*-menthol 20 mg in 2 ml-reaction mixture was converted into the corresponding  $\alpha$ -MenG 42 mg with 99% yield accompanying accumulation of its crystals. In comparison with  $\alpha$ -anomer-selective glucosylation of *l*-menthol using the lyophilized cells of *X. campestris* WU-9701 (the previous study), the reaction time for  $\alpha$ -MenG production to give the same molar conversion yield (99%) was drastically shortened from 48 h for the previous study to 3 h for the present study. Therefore, the author succeeded in construction of an efficient  $\alpha$ -MenG production system using the recombinant XgtA, in other words the recombinant *E. coli* cells expressing highly *xgtA* as the enzyme source. On the

other hand, the author succeeded in also  $\alpha$ -anomer-selective glucosylation of hydroquinone using the recombinant XgtA with shortened reaction times. In conclusion, the author succeeded in construction of an efficient  $\alpha$ -glucoside production system using recombinant XgtA produced by *E. coli* expressing highly *xgtA* as the enzyme source.

In chapter 8, the studies done in this thesis are summarized and concluded.

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### AWARDS

None

## 研 究 業 績

種 類 別	題 名、 発 表 ・ 発 行 掲 載 誌 名、 発 表 ・ 発 行 年 月、 連 名 者
1. 論文 ○ (報文)	1. Identification of the Gene Encoding a Novel $\alpha$ -Glucosyl Transfer Enzyme from <i>Xanthomonas campestris</i> WU-9701 and Its Application to $\alpha$ -Anomer-Selective Glucosylation of Menthol, in press. <u>Toshiyuki Sato</u> , Jun Saito, Keishiro Yoshida, Takanori Tsugane, Susumu Shimura, Kuniki Kino, and Kohtaro Kirimura.
(報文)	2. Enzymatic Synthesis of $\alpha$ -Menthyl $\alpha$ -Maltoside and $\alpha$ -Menthyl $\alpha$ -Maltooligosides from $\alpha$ -Menthyl $\alpha$ -Glucoside by Cyclodextrin Glucanotransferase, J. Biosci. Bioeng., Vol. 94, No. 2, 119-123, 2002年9月. Hiroyuki Do, <u>Toshiyuki Sato</u> , Kohtaro Kirimura, Kuniki Kino, and Shoji Usami.
○ (報文)	3. Enzymatic Synthesis of $\alpha$ -Arbutin by $\alpha$ -Anomer-Selective Glucosylation of Hydroquinone Using Lyophilized Cells of <i>Xanthomonas campestris</i> WU-9701, J. Biosci. Bioeng., Vol. 93, No. 3, 328-330, 2002年3月. Jun Kurosu, <u>Toshiyuki Sato</u> , Keishiro Yoshida, Takanori Tsugane, Susumu Shimura, Kohtaro Kirimura, Kuniki Kino, and Shoji Usami.
○ (報文)	4. $\alpha$ -Anomer-Selective Glucosylation of (+)-Catechin by the Crude Enzyme, Showing Glucosyl Transfer Activity, of <i>Xanthomonas campestris</i> WU-9701, J. Biosci. Bioeng., Vol. 90, No. 6, 625-630, 2000年12月. <u>Toshiyuki Sato</u> , Hiroyuki Nakagawa, Jun Kurosu, Keishiro Yoshida, Takanori Tsugane, Susumu Shimura, Kohtaro Kirimura, Kuniki Kino, and Shoji Usami.

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2. 講演	<p>1. Application of Recombinant E. coli Cells Expressing the Gene Encoding <math>\alpha</math>-Glucosyl Transfer Enzyme of <i>Xanthomonas campestris</i> WU-9701 for <math>\alpha</math>-Anomer-Selective Glucosylation of Menthol, 3rd European Symposium on Enzymes in Grain Processing, Leuven, Belgium, 2002年9月, (Abstract P. 92) Kohtaro Kirimura, <u>Toshiyuki Sato</u>, Jun Saito, and Kuniki Kino.</p> <p>2. <math>\alpha</math>-Anomer-Selective Glucosylation of (+)-Catechin and Hydroquinone Using <math>\alpha</math>-Glucosyl Transfer Enzyme of <i>Xanthomonas campestris</i> WU-9701, 3rd European Symposium on Enzymes in Grain Processing, Leuven, Belgium, 2002年9月, (Abstract P. 93) <u>Toshiyuki Sato</u>, Kuniki Kino and Kohtaro Kirimura.</p> <p>3. <i>Xanthomonas campestris</i> WU-9701が生産する新規グルコース転移酵素の遺伝子クローニング、 日本化学会第81春季年会、東京、2002年3月、 (講演要旨集 p. 900 ) <u>佐藤利行</u>、<u>齋藤淳</u>、<u>桐村光太郎</u>、<u>木野邦器</u>、<u>宇佐美昭次</u>。</p> <p>4. 新規清涼剤としての <math>\alpha</math>-メントール <math>\alpha</math>-マルトシドの酵素的合成、 日本化学会第81春季年会、東京、2002年3月、 (講演要旨集 p. 900 ) <u>長谷川敦一</u>、<u>堂裕行</u>、<u>佐藤利行</u>、<u>桐村光太郎</u>、<u>木野邦器</u>、<u>宇佐美昭次</u>。</p>



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	<p>1 0 . <math>\alpha</math>-Anomer-Selective Glucosylation of <math>\alpha</math>-Menthol and (+)-Catechin Using <i>Xanthomonas campestris</i> WU-9701, 2000 International Chemical Congress of Pacific Basin Societies, Honolulu, U. S. A., 2000年12月, (Abstract Part 1 3・502) <u>Toshiyuki Sato</u>, Keishiro Yoshida, Takanori Tsugane, Susumu Shimura, Kohtaro Kirimura, Kuniki Kino, and Shoji Usami.</p> <p>1 1 . <i>Xanthomonas campestris</i> WU-9701由来の酵素を用いたヒドロキノンの <math>\alpha</math>-アノマー選択的グルコシル化による <math>\alpha</math>-アルブチンの合成、日本生物工学会、札幌、2000年8月、(講演要旨集 p. 231) 黒須潤、<u>佐藤利行</u>、桐村光太郎、木野邦器、宇佐美昭次.</p> <p>1 2 . <i>Xanthomonas campestris</i> WU-9701由来の <math>\alpha</math>-アノマー選択的グルコシル化を触媒する <math>\alpha</math>-glucosidaseの精製および酵素的諸性質の検討、日本生物工学会、札幌、2000年8月、(講演要旨集 p. 231) 熊田有未、<u>佐藤利行</u>、吉田圭司郎、津金貴則、志村進、桐村光太郎、木野邦器、宇佐美昭次.</p> <p>1 3 . <i>Xanthomonas campestris</i> WU-9701由来の酵素を用いたカテキン <math>\alpha</math>-グルコシドの <math>\alpha</math>-アノマー選択的合成、日本化学会第78春季年会、千葉、2000年3月、(講演要旨集 p. 793) <u>佐藤利行</u>、中川博之、桐村光太郎、木野邦器、宇佐美昭次.</p>

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	<p>18. 結晶蓄積型新規微生物反応によるメントール配糖体のアノマー選択的合成、日本化学会第76春季年会、神奈川、1999年3月、（講演要旨集 p.1215） 中川博之、佐藤利行、土橋幸生、神垣清威、桐村光太郎、宇佐美昭次.</p> <p>19. 凍結乾燥菌体を利用したメントールのアノマー選択的グルコシル化、日本生物工学会、広島、1998年9月、（講演要旨集 p.134） 土橋幸生、中川博之、佐藤利行、吉山正章、吉田圭司郎、志村進、桐村光太郎、宇佐美昭次.</p>

## Acknowledgement

This thesis is a collection of studies which have been carried out under the direction of Prof. Dr. Kohtaro Kirimura, Laboratory of Applied Biochemistry, Department of Applied Chemistry, School of Science and Engineering, Waseda University during the period from 2000 to 2003. The author would like to express a sincere gratitude to Prof. Kohtaro Kirimura for his helpful and useful advice and continuous encouragement throughout this work. The author also expresses a gratitude to him for providing the opportunity to present this thesis to Waseda University.

Grateful acknowledgement is made to Prof. Dr. Hiroyuki Nishide for helpful and useful advice and continuous encouragement throughout this work.

Grateful acknowledgement is made to Prof. Dr. Kuniki Kino for helpful and useful advice and continuous encouragement throughout this work.

Grateful acknowledgement is made to Emeritus Prof. Dr. Shoji Usami for helpful and useful advice and continuous encouragement throughout this work.

The author is much indebted to all collaborators involved in the works of this thesis done for past three years. The author expresses gratitude to the members of Enzyme Creative Molecular Engineering group as well as all the members of Laboratory of Applied Biochemistry.

The author expresses a sincere gratitude to Dr. Yoshitaka Ishii, Mr. Kiyotake Kamigaki, Mrs. Takako Murakami-Nitta, and Mr. Toshiki Furuya for their generous encouragement and helpful and useful advice.

Finally, the author expresses his deepest gratitude to his parents for their generous support with much tolerant encouragement.

Toshiyuki Sato