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Biological Activity of Enzymatically Synthesized Polyphenol Glycoside on Microbial Growth

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ABSTRACT

Biological Activity of Enzymatically Synthesized Polyphenol Glycoside on Microbial Growth. We have studied an indigenous bacterial strain produced a glycosyl transfer enzyme (CGTase) yielding polyphenol glycosides from a substrate of starch and polyphenol-aglycone. We observed that the CGTase derived from culture filtrate of some microbial strains (*Candida rugosa*, *Bacillus megaterium*, *B. coagulans* and *B. polymixa*) could synthesize transfer products in the presence of appropriate polyphenol-aglycones as their acceptors. An inhibitory effects of enzymatically synthesized polyphenol glycosides against bacterial growth was furthermore examined. It was found that polyphenol-glycoside, as one of the transfer products, exhibited high antibacterial activity on the growth of *Bacillus subtilis* and *Escherichia coli*, no effect when on *Bacillus cereus*.

Key words : Cyclodextrin glucano transferase (CGTase), enzymatic transglycosylation, polyphenol glycoside, antibacterial activity.

INTRODUCTION

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) catalyzes a reversible conversion of polysaccharides and polyphenolic compounds to polyphenol glucosides (Funayama *et al*., 1993). Chemical synthesis of such kinds polyphenol glucosides is not easy because it results in the production of glycosides mixture in α- and β-configuration (Nilson, 1988). Polyphenols, such as hydroquinone, pyrocathecol, resorcinol and catechin, are considered to have strong biological activities. Polyphenols are used as an antipruritic or antiseptic in cosmetics (Akiu *et al*., 1988), as an inhibitor of melanogenesis (Palumbo *et al*., 1991), as an antioxidant (Ioku *et al*., 1992), as a bacterial growth inhibitor (Ahn *et al*., 1991) or as a potent antimutagent (Dinoto & Sulistyo, 2000). However, polyphenols uses are limited since they are easily degraded in an aqueous solution resulting in rapid browning (Kitao & Sekine, 1994). In the case of hydroquinone, the main transfer product, identified as hydroquinone β-glucopyranosides (Sulistyo *et al*., 2000), was much more resistant than that of aglycone-hydroquinone to light irradiation (Kitao *et al*., 1995). It was suggested that some physical properties of the polyphenol glycoside were improved and may become an antioxidant, antimutagent, antimelanogenesis and antitoxic agent through en-

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zymatic transglycosylation reaction (Sulistyo *et al*., 2000; 2001a & 2001b). In this study, we examined the transglycosylation reaction catalyzed by CGTase of *Bacillus coagulans* in the presence of soluble-starch as the substrate and resorcinol as the acceptor, and furthermore the inhibitory activity of enzymatically synthesized polyphenol glycosides on bacterial growth was also studied.

MATERIALS AND METHODS

Crude Enzyme preparation

The crude CGTase of bacterial strains were prepared by autolysis of intact cells grown in a media containing 2.0% soluble starch 0.5% pepton, 0.1% K_2HPO_4, 0.05% NaCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.0001% $ZnSO_4$, 0.0001% $CuSO_4$, 0,0001% $MnSO_4$, and incubated at room temperature for 5 days. The intact cells were washed with water and left to autolyze for 24h. The autolyzate was centrifuged and the supernatant obtained was dialyzed against 50mM citrate buffer (pH 6.5). The dialyzed suspension was used as a crude (CGTase) enzyme preparation (Sulistyo & Soeka, 2000).

Assay of enzyme activity

The CGTase activity was assayed by spectotrophotometric measurement using soluble starch as the substrate (Masataka *et al*., 1995). One unit of enzyme activity was defined as the amount of enzyme which reduced 0.5 the unit absorbance at 660 nm per minute under the reaction condition. The reaction mixture (450 µl), containing 0.5% soluble starch and the crude enzyme in 50 mM citrate buffer (pH 6.5) was incubated at 50^{o}C for 10 minutes. The reaction was stopped with 1.0 ml of 0.5 N HCl, before addition of 2.5 ml of 0.05% KI solution containing 0.005% I_2. The absorbance at 660 nm was measured using a UV-Vis spectrophotometer (Perkin-Elmer Lamda 3B), after keeping the reaction mixture at room temperature for 20 min.

Thin-layer chromatography

Thin layer chromatography **(**TLC) was carried out with the ascending method, using silica gel 60 plates (Kiesel Gel-Merck) and ethyl acetate-acetic acid-water (3 : 1 : 1, v/v) as solvent. Spots were detected by spraying with 20% H_2SO_4 in methanol and subsequent heating at 100-150 ^{o}C. The retention factor (*Rf*) of the spots can be measured by measuring the ratio between distance traveled by substance and the distance traveled by solvent.

Preparation and purification of resorcinol glycoside

Crude CGTase was added to 100 ml of a 50 mM sodium acetate buffer (pH 6.5) containing 5.0% soluble starch and aglycones of 2.5% (hydroquinone, pyrocatechol, resorcinol) or 1.0% (catechin). To isolate the transfer product, a reaction mixture (100 ml) was then concentrated *in vacuo* to 10ml. The concentrate was applied to a column of ODS Wakosil 25 that had been equilibrated with distilled water containing 0.1% formic acid in methanol, and the products were eluted with gradient concentration of methanol up to 100%. The fractions containing glycosides were collected and then analyzed by TLC.

Assay of inhibition on bacterial growth

Minimum inhibitory concentration (MIC) was measured using a serial two-fold dilution method. Serially diluted sample solutions (0.1 ml) were added to

9.9 ml of sterile medium containing 1.5% agar at around 45ºC in a petri dish. This mixture was thoroughly mixed and then solidified. One loop of suspension of the test microbial strains those were precultured at 37ºC for 48 h, were then inoculated onto agar medium and incubated at 37ºC for 24 to 72 h. The MIC was identified by comparing visually the microbial growth. To examine the sensitivity of microbial strains towards resorcinol glycosides as antibacterial component, efficacy is determined by measuring the diameter of the inhibition zone of the components that results from diffusion of the component into the medium surrounding filter-paper discs are impregnated with antibacterial component and placed on the surface of agar plate that has been seeded with the bacterial strains to be tested.

RESULTS AND DISCUSSION

The production of extracellular CGTase of four tested strains (*B. megaterium, C. rugosa, B. coagulans* and *B. polymyxa*) is shown in Figure 1. The microbial CGTases of the tested strains were optimally extracted after 4 to 5 days of incubation and becoming decreased down after incubating for 6 days. The CGTase was produced in significant amount when soluble starch was used as a carbon source. The optimum activity of CGTase of *B. megaterium, C. rugosa B. coagulans,* and *B. polymyxa* were 3.1 U/ml, 2.7 U/ml, 2.4 U/ml and 2.3 U/ml, respectively.

The optimum pH of CGTase activities of the tested strains were observed by measuring the enzyme activities toward the soluble starch from pH 3.0 to 8.0. The optimal activity of CGTase of *C. rugosa* was at pH 6.0 (7.2 U/ml), *B. coagulans,* and *B. polymyxa* were at pH 7.0 (2.7 and 2.2 U/ml, respectively) and *B. megaterium* was at pH 6.5 (4.2 U/ml) (Figure 2). The CGTase of *C. rugosa, B. coagulans* and *B. megaterium* hydrolyzed the soluble starch with the highest rate at 50ºC (7.6 U/ml, 2.9 U/ml and 3.8 U/ml, respectively) while *B. polymyxa* was at at 55ºC (1.8 U/ml) (Figure 3).

The polyphenol glycosides were synthesized by incubating 5% soluble starch and 1.0-2.5% polyphenol-aglycone as acceptors with bacterial filtrate containing CGTases of tested strains at optimum temperature and pH (50 to 55EC at pH 6.0 to 7.0) for 24 h. To scale up production of polyphenol glycoside, 5% soluble starch and 2.5% resorcinol-aglycone was incubated with bacterial filtrate of *B. coagulans* at 50EC and pH 7.0 for 24 h, since the synthesis of polyphenol glycoside by transglycosylation reaction of *B. coagulans* has not reported yet. Since the reaction products contained some transfer products mixed with hydrolysis products, amyloglucosida-se was furthermore added to the reaction mixture in order to convert oligoglucosides to monoglucoside.

Figure 4, shows a thin layer chromatogram of the reaction products. The Rf value of detected catechin-glucoside as the transfer product was exhibited at Rf 0.85 (TP-2) and Rf 0.87 (TP-1). This was comparable to the Rf value of arbutin (0.87) as the authentic standard of polyphenol glucoside. Purification were carried out to isolate the transfer product from the reaction mixture (RM) as described in the MATERIALS AND METHODS. Most of the transfer products (TP-1, TP-2, TP-3) were found in water layer (PGwl) after following extraction with diethyl ether (PGbp), and most of residual acceptor was with small

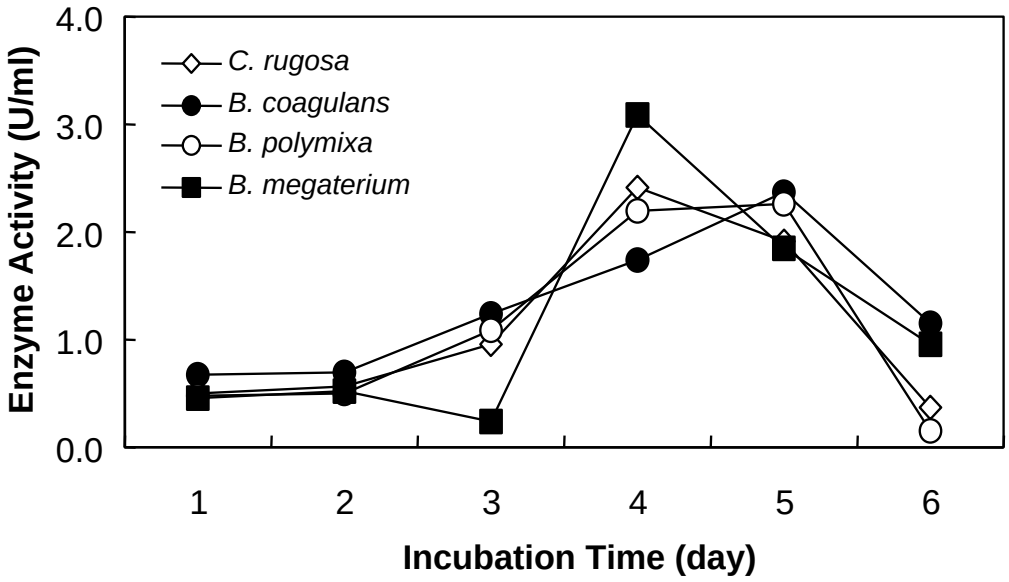

Figure 1. Capability of isolated strains on production of CGTase.

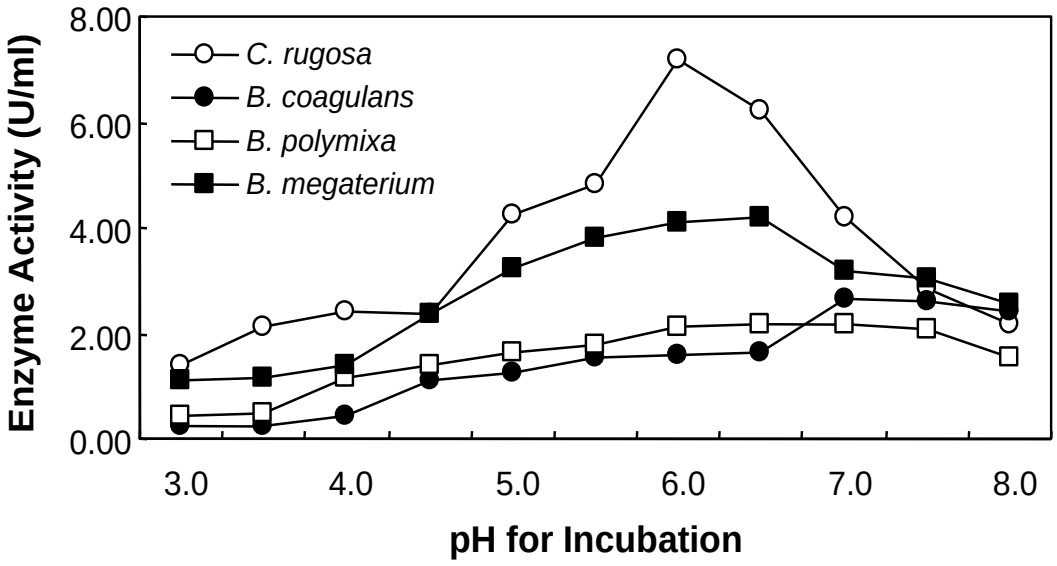

Figure 2. Effect of pH on enzymatic activity of microbial.CGTase.

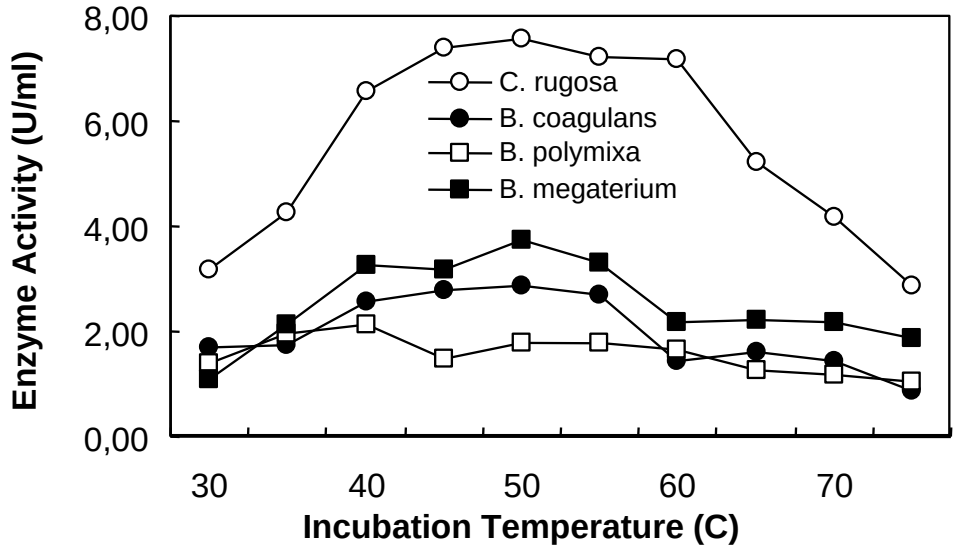

Figure 3. Effect of temperature on enzymatic activity of microbial CGTase

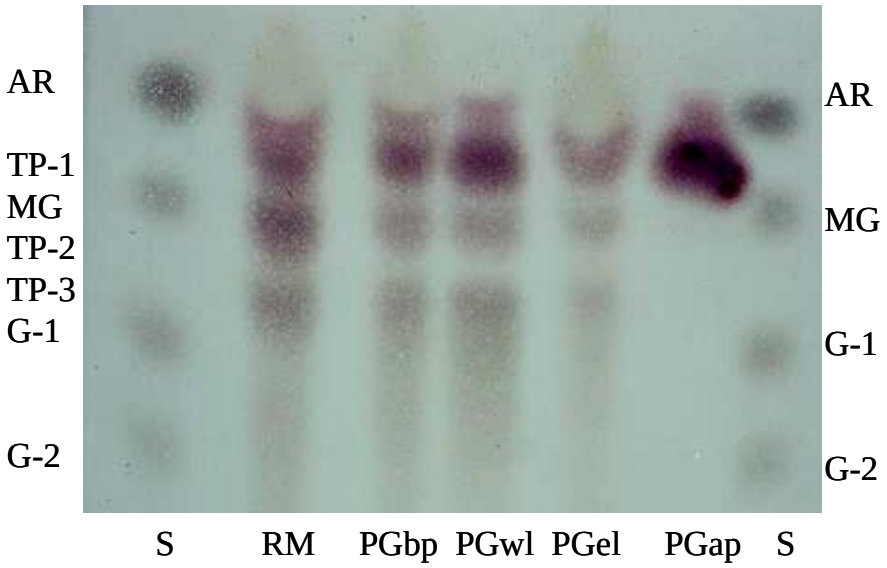

Figure 4.Thin layer chromatogram of transglycosylation reaction products.
Transfer products (TP-1, TP-2, TP-3) before purified (PGbp) and after purified (PGap) were detected in the presence of aglycone in accordance to standard solution (S) containing Maltose (G-2); Glucose (G-1); Methyl □-glucoside (MG); Arbutin (AR).

amount of the transfer product solved into ether layer (PGel). Single spot of polyphenol glucoside (PGap) was finally obtained after purification by ODS column chromatography eluted with gradient concentration of methanol

Purification of the reaction products were carried out to isolate the transfer product from the reaction mixture as described in the Materials and Methods. Most of the transfer product was found in water layer after following extraction with diethyl ether and most of residual acceptor was with small amount of the transfer product solved into ether layer. Single spot of catechin glucoside was finally obtained after purification by ODS column chromatography eluted with gradient concentration of methanol.

The inhibitory activities were monitored by counting the growth of colonies. The inhibitory activities of polyphenol glycoside at low to high concentration (44.9 to 89.7 ppm) were effective to inhibit the growth of *B. subtilis* and *E. coli*, but was ineffective to inhibit *B. cereus*. Otherwise both of arbutin and polyphenol-aglycone were not effective to inhibit the growth of *B. subtilis* and *E. coli*, neither to *B. cereus* those indicated that polyphenol glycoside exhibited higher inhibitory activity on bacterial growth rather than commercial glycoside and polyphenol-aglycone. However, nor glycosides neither aglycone ones effectively inhibited the growth of *B. cereus*. It was suggested that as a bacterial toxin producer in foodstuff, *B. cereus* could produce antitoxical agent that could detoxicate any toxical as well as antibacterial agents which was derived from other else (Table 1).

Table 1. Inhibitory concentrations of polyphenolic compounds on bacterial growth.

Microbial Strains	Arbutin (ppm)			Glycoside (ppm)			Aglycone (ppm)		
	44.90	67.30	89.70	44.90	67.30	89.70	44.90	67.30	89.70
Bacillus subtilis	-	-	-	+	++	++	-	-	-
Escherichia coli	-	-	-	+	++	++	-	-	-
Bacillus cereus	-	-	-	-	±	±	-	-	-

In the course of examining the inhibition activity of polyphenol glycosides on microbial growth, we investigated the MIC of each aglycones and polyphenol glycosides against 21 microbial strains, those were *Bacillus firmus, B. subtilis, B. megaterium, B. cereus, B. coagulans, Enterobacter liquefaciens, E. cloacae, Pseudomonas aeruginosa, P. stutzeri, P. fluorescens, Candida rugosa, C. brumtii, C. rhagii, C. rekaufii, C. tropicalis, Saccharomyces elipsoides, S. cereviciae, Streptococcus thermophilus, Aspergillus niger, Penicillium expansum* and a strain of yeast. The inhibitory activities were monitored by counting the growth of colonies. The inhibitory activities of polyphenol glycosides at high concentration (800 ppm) were comparable to those of arbutin but slightly higher than those of polyphenol-aglycone. They were active at higher concentrations (800 ppm) against most of tested microbial strains, however, varied in their inhibitory activities against few tolerance strains. Arbutin was more active against *C. rugosa, B. coagulans, E. liquefaciens, B. subtilis* and *P. stutzeri*, however, *it was less active against S. cereviciae, A. niger*

and *C. tropicalis.* Somehow, aglycone-polyphenol was moderately active against some strains, but it was less active against *E. liquefaciens, P. stutzeri, C. rekaufii* and *C. tropicalis*. Otherwise, polyphenol glucoside was more active against most of all the tested microbial strains, however, it was less active against *S. cereviciae* and *P. expansum*, and slightly active against *C. tropicalis* (Table 2).

Tabel 2. Effect of polyphenolic concentration on microbial growth.

Microbial	Arbutin (ppm)				Glycoside (ppm)				Aglycone (ppm)			
Strains	200	400	600	800	200	400	600	800	200	400	600	800
B. firmus	+	+	+	+	++	+	+	+	-	+	+	+
B. subtilis	+	++	++	++	-	+	+	+	-	-	+	+
B. megaterium	+	++	++	+	-	++	+	+	-	+	+	+
B. cereus	+	+	+	+	+	+	+	+	+	+	+	+
B. coagulans	++	++	+	+	+	+	++	+	-	+	+	+
E. liquefaciens	+	++	+++	+	+	+	+	+	-	-	-	-
E. cloacae	+	+	+	+	+	+	+	+	+	+	+	+
P. stutzeri	++	++	+	+	+	+	+	+	-	-	-	-
P. fluorescens	-	-	+	+	-	+	+	+	-	+	+	+
C. rugosa	++	++	++	+++	+	+	+	+	-	-	-	-
C. brumtii	+	+	+	+	+	+	+	+	-	+	+	+
C. rhagii	-	-	-	+	-	+	+	+	+	+	+	+
C. rekaufii	+	+	+	+	-	+	+	+	-	-	-	-
C. tropicalis	-	-	-	-	-	-	-	-	-	-	-	-
S. elipsoides	-	-	-	+	+	+	+	+	+	+	+	+
S. cerevisiae	-	-	-	-	-	-	-	-	+	+	+	+
S. thermophilus	-	-	-	-	+	+	+	+	+	+	+	+
A. niger	-	-	-	-	+	+	+	+	+	+	+	+
P.expansum	-	-	-	+	-	-	-	-	+	+	+	+
Inhibited strains	12	12	13	16	12	17	17	17	9	14	15	15

(+++), highly inhibited; (++), moderately inhibited; (+), slightly inhibited; (–), no inhibition

CONCLUSION

Polyphenol glycoside could be synthesized by application of transglucosylation of CGTase of indigenous microbial strains from polysaccharides and polyphenol-aglycone. The transfer products can be determined by TLC, and furthermore purified using ODS column chromatography eluted with gradient concentration of methanol. Polyphenol glycoside had shown higher antimicrobial activity than that of arbutin and polyphenol aglycone itselves. The antimicrobial activity of the Polyphenol glycoside was found to be increased since linkage position of glycoside could increase its stabililty, and resulted in higher hydroxyl moieties than that of arbutin as a commercial polyphenol glycoside.

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