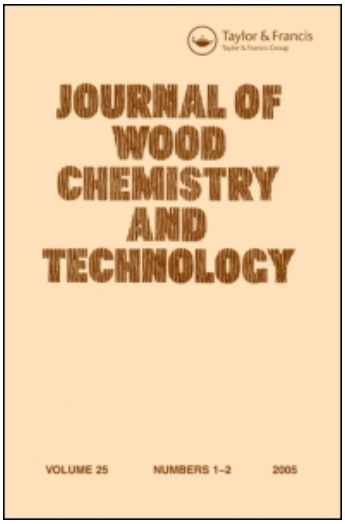

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Inhibitory Effects of Bark Proanthocyanidins on the Activities of Glucosyltransferases of *Streptococcus Sobrinus*

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INHIBITORY EFFECTS OF BARK PROANTHOCYANIDINS ON THE ACTIVITIES OF GLUCOSYLTRANSFERASES OF *STREPTOCOCCUS SOBRINUS*

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ABSTRACT

Seventy percent acetone aqueous extracts of *Acacia mearnsii* and *Larix* spp. bark inhibited the activity of glucosyltransferase (GTase) derived from *Streptococcus sobrinus* to a greater degree than green tea or oolong tea extracts. The GTase inhibitory activity increased as the molecular weight of proanthocyanidins contained in the extracts increased. The GTase inhibition was very closely related to the hydroxylation patterns in the B-rings of synthesized proanthocyanidins, where compound with a catechol ring showed high activity than with a pyrogallol B-ring, and it was a non-competitive type of inhibition.

INTRODUCTION

Present-day accumulation of bark reaches 5.6 million tons per year in Japan[1], but most of it is not utilized and is regarded as a troublesome product in the lumber industry. It is however needless to say that bark is a very important tissue

for the protection of a tree from external invasion by wood-rotting fungi or insects, and that polyphenols (proanthocyanidins) contained in the bark are believed to play an important role in these defensive actions.[2] This is based on the properties of denatured proteins or of inhibited enzymes by polyphenols. We attempted to attain the effective utilization of bark by considering these characteristics of the polyphenols.

In regard to the enzyme inhibition caused by polyphenols, the inhibitory effects on glucosyltransferase (GTase) by the polyphenols contained in green and oolong tea extracts have been reported recently.[3-5] Dental caries are developed by the degradation of water-insoluble glucans synthesized from sucrose by GTase which is derived from *Streptococcus sobrinus* or *S. mutans*.[6] The applications of these tea polyphenols as a preventative for dental caries in foods and sanitary supplies are currently available in some consumer products in Japan. The effective polyphenols in green tea for GTase inhibition are mainly monomeric polyphenols having galloyl groups, and those in oolong tea are enzymatically oxidized compounds[7] produced by semifermentation of green tea polyphenols. On the other hand, bark polyphenols are mainly proanthocyanidins consisting of polyhydroxyflavan-3-ol repeating units linked through C4-C6 or C4-C8 bonds.[8] In this paper, the GTase inhibition ability of some bark proanthocyanidins and the correlation between the chemical structure of proanthocyanidins and their inhibition activity are discussed.

EXPERIMENTAL

Preparations of proanthocyanidins

Green tea (GTE), oolong tea (OTE), and commercially available Quebracho (*Schinopsis lorentzii*) extracts (QE) were extracted with 70 % aqueous acetone. The extracts of Acacia (*Acacia mearnsii*) bark (ABE), Karamatsu (*Larix* spp.) bark (KBE) with 70 % aqueous acetone were fractionated by LH-20 gel column chromatography using ethanol, methanol, and 70% aqueous acetone as eluents. These ethanol, methanol, and 70% aqueous acetone eluted fraction was termed EE, ME, and AE, respectively as described in a previous paper.[9] Proanthocyanidins

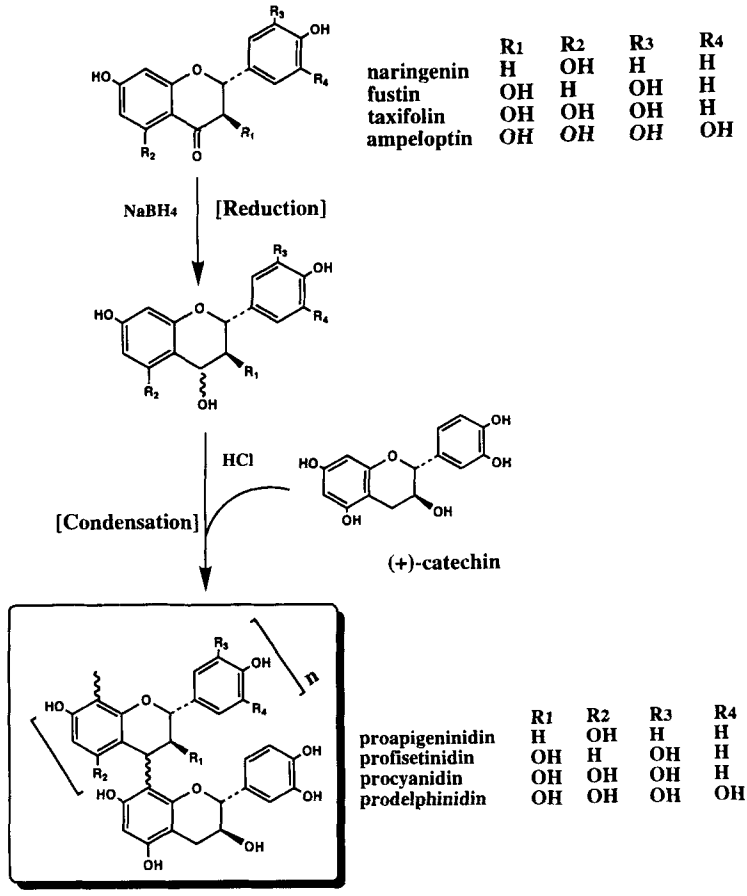


FIGURE 1. Synthesis scheme of proanthocyanidins

were synthesized non enzymatically according to the method in our previous paper.[10] The procedure of the synthesis is shown in Fig. 1. Taxifolin, fustin, and ampeloptin were obtained from the heartwood of *Larix leptolepis* , *Rhus succedanea* L, and *Salix sachalinensis* Fr. Schm, respectively. Naringenin was purchased from Wako Pure Chemical Industries LTD. These flavanoids were reduced with sodium borohydride to obtain 4-hydroxyflavan derivatives. Procyanidin (PC), profisetinidin (PF), prodelfinidin (PD), and proapigeninidin

(PA) oligomers were synthesized by condensation of the corresponding 4-hydroxyflavan derivatives with (+)-catechin under acidic conditions. These oligomers were separated by Sephadex LH-20 gel column chromatography in a similar manner as that performed on the bark extracts.

Analytical methods for polyphenols

The polyphenol analyses were conducted by using the following specific coloration reactions: the Folin-Denis method[11] for the determination of total phenol content and vanillin-HCl method[12] to determine proanthocyanidin content. Furthermore, tannin content was determined by the Lowenthal method[13] using a redox reaction. Gel permeation chromatography (GPC) was recorded with a JASCO TRIROTAR system with Shodex GPC columns KF-802 and KF-804 (4.6 mmϕ x 250 mm) using tetrahydrofuran as an eluent. The chromatogram was calibrated against standard polystyrenes (Molecular weights: 2000 and 9000), (+)-catechin, and synthesized PF dimer. Average of molecular weights (Mn, Mw) were calculated by an integrator, Jasco 807-IT, from the molecular weight distributions obtained by the GPC measurement.

Phenyl nucleus exchange reaction (NER)

NER was carried out in a 2 ml glass ampoule with 10 mg of polyphenol sample and 500 μl of the degradation reagent at a temperature of 80°C or 150 °C. The constitution of the degradation reagent was benzene : phenol : BF_3-phenol complex = 10 : 19 : 3 (v/v). After the reaction, the reaction vessel was cooled to room temperature and the reaction mixture was transferred quantitatively into a beaker with ethyl ether. A known amount of internal standard (dibenzyl in benzene) was added. Ether insoluble materials were filtered and washed with ethyl ether. The filtrate and washings were combined in a separatory funnel to which an excess of saturated brine was added. After the extraction (30 ml x 3), the ether layer was dried over sodium sulfate and evaporated up to a small volume (4-5 ml). One-hundred μl of the ether solution was transferred into a small vial to

which a few drops of pyridine and 100μl of N,O-bis(trimethylsilyl) acetamide were added. After one hour derivatization reaction at room temperature, the TMS derivatives were analyzed quantitatively using gas liquid chromatography (GLC). GLC was performed by a Yanagimoto G-180 using a methyl silicone capillary column (Guadrex S2006, 0.25 mm I.D. x 25 m length x 0.25 mm film thickness). Column temp. of GLC was maintained at 80 °C for 2.5 min. and then increased to the final temp. of 250 °C at a rate of 2 °C/min .

Preparation of GTase

Streptococcus sobrinus 6715 was grown for 16 hr at 37 °C in 5 l of Todd Hewitt (TH) broth. After centrifugation of the liquid medium at 5000 rpm for 15 min, the cells were collected and then extracted with 75 ml of 8M urea at 20 °C for 1 hr with stirring. The crude enzyme solution containing urea was dialyzed against 10 mM potassium phosphate buffer (pH 6) until the urea was removed entirely. One ml of the crude enzyme solution was pipetted into a microtube and stored in a freezer at -80 °C.

Assay for GTase inhibitory activity

Insoluble glucan synthesized by GTase was measured turbidimetrically with a spectrophotometer (Jasco V-520) by determining the increase in A_{550}. GTase was incubated in 3 ml of 0.1 M phosphate buffer (pH 6.0) containing 1 % sucrose, 0.1 % sodium azide, 0.5 % dextran T-10, and in the presence or absence of polyphenols at 37 °C for 3 hr. The volume of the crude GTase solution used in the assay was determined by that giving an absorbance of 1.0 at 550 nm. Inhibition rate is expressed by the following equation: Inhibition rate (%) = 100 x (Ac-Ap)/Ac (Ac and Ap represent absorbance obtained in the control and in the polyphenol dose, respectively.) IC_{50} means the polyphenol concentration (μg/ml) giving 50 % inhibition of GTase. The EE, ME, and AE fractions of Acacia, Karamatsu, and synthesized proanthocyanidins were tested for the IC_{50} activity.

TABLE 1 Polyphenol analyses of teas and barks extracted by 70% aqueous acetone.

Extracts	Analysis methods		
	Total phenols	Flavanoids	Tannins
GTE	40.3	49.5	5.2
OTE	42.5	42.2	29.5
ABE	66.5	57.3	45.5
KBE	70.2	66.1	53.9

GTE : Green tea extracts OTE : Oolong tea extracts
ABE : Acacia bark extracts KBE : Karamatsu bark extracts

These figures show the weight % of the amounts caluculated by the calibration curves using (+)-catechin to that of the extracts used.

RESULTS AND DISCUSSION

Inhibitory activity of the bark polyphenols

Table 1 shows the data obtained by the methods of Folin-Denis, vanillin-HCl, and Lowenthal for 70% aqueous acetone extracts from teas and barks. In general, the Folin-Denis method is used for total phenols, and the vanillin HCl assay is widely employed as a method for quantitative determination of flavanols. Additionally, the Lowenthal method is employed for the quantitative analysis of tannins which are capable of forming precipitates with gelatin. In this paper, proanthocyanidins (condensed tannins) are in conformity with those substances. Flavanol contents of GTE and OTE are very similar, but their proanthocyanidin contents are quite different. Because GTE contains mainly monomeric polyphenols, it does not exhibit tanning characteristics. The results from the Lowenthal method with ABE and KBE are about 80% of the values of the vanillin-HCl method, which indicates that most of the polyphenols in bark extracts consist of proanthocyanidins. The relationship between the concentration of these extracts and their inhibition rate on GTase is shown in Fig. 2. Both bark extracts exhibited higher inhibition rates than GTE or OTE. Particularly KBE

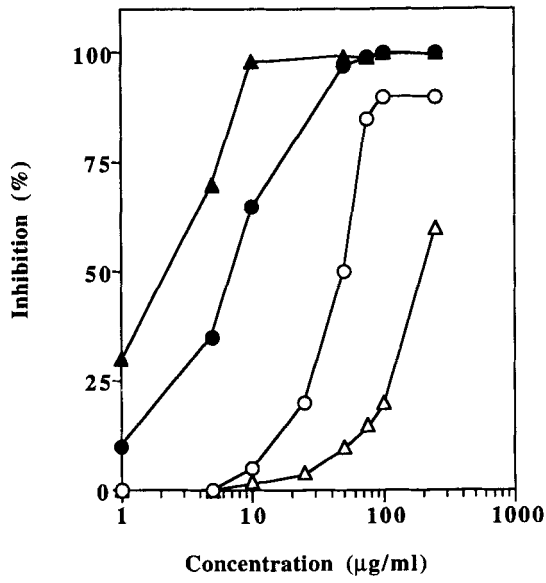


FIGURE 2. GTase inhibitory activity of 70 % acetone extracts from teas and barks.

Legend : ▲ Karamatsu bark ● Acacia bark
○ Oolong tea △ Green tea

showed very high inhibition rate, and its IC_{50} value was about 2.0 μg/ml. Judging from these IC_{50} values, the inhibitory effect of KBE was about ten times as high as that of OTE and forty times that of GTE. Therefore, the inhibitory activity of these extracts was presumed to relate to the contents and structures of proanthocyanidins.

Influences of the molecular weight and phenyl nucleus of bark proanthocyanidins on the inhibitory activity of GTase

The molecular weight of each eluate fraction separated by LH-20 gel column chromatography and their IC_{50} values for GTase are shown in Table 2. The

TABLE 2 The influences of molecular weight of fractionated bark extracts on GTase inhibition activity.

Sample	$\overline{Mn}$	$\overline{Mw}$	Dp	IC_{50} (μg/ml)
ABE-EE	355	720	2.4	250
-ME	1045	1495	3.9	15.0
-AE	2474	4235	11.4	1.7
KBE-EE	325	750	2.2	13.0
-ME	1150	1585	4.0	5.0
-AE	2852	4825	13.5	1.0

$\overline{Mn}$: Number avarage molecular weight $\overline{Mw}$: Weight average molecular weight

Dp: Degree of polymerization

IC_{50}: The concentration (μ g/ml) giving 50% inhibition of GTase.

Sample name: refer to experimental section

molecular weight of these fractions increased in the following order, EE, ME, and AE fraction. Judging from the molecular weight of the top peak of GPC, these fractions consist of dimer to trimer, trimer to pentamer, and greater than hexamer, respectively. As shown in Fig. 3, their IC_{50} values showed a tendency to decrease with an increase of the molecular weight of the polyphenols, and the rapid increase of inhibitory effect was shown in the neighborhood of average molecular weight 1500 corresponding to pentamer to hexamer of proanthocyanidins.

The Nucleus exchange reaction (NER) is a method designed for the analysis of phenolic nuclei constituting lignin[14] and proanthocyanidins.[15] In the case of condensed tannins, phloroglucinol or resorcinol from the A-ring, and catechol or pyrogallol from the B-ring are liberated in large yields. Considering the yields of degradation products, the NER gives more information with regard to the phenolic nuclei constituting proanthocyanidins than the other degradation methods such as butanol-hydrochloric acid[16] or thiolysis.[17] The amounts of nuclei liberated by NER of the natural polyphenol fractions are shown in Table 3. On ABE, phloroglucinol and resorcinol nuclei were liberated in a molar ratio of about

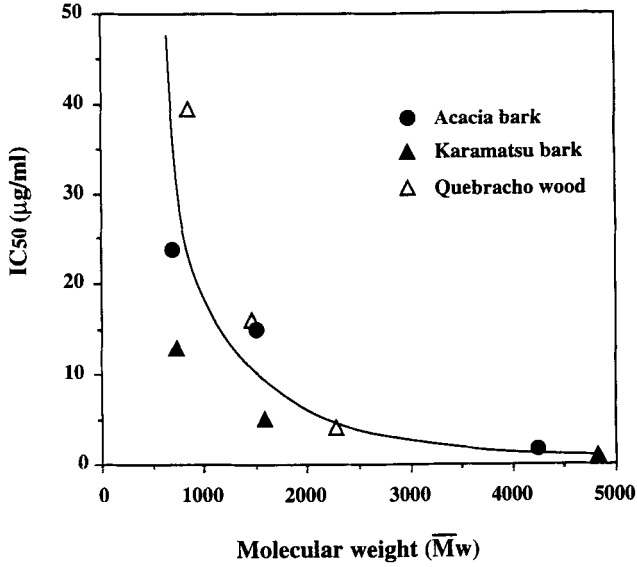


FIGURE 3. The relationship between molecular weight of polyphenols separated by LH-20 column chromatography and their GTase inhibitory activity.

IC50 : see Table 2

1 : 3 from A-ring, and catechol and pyrogallol nuclei in a molar ratio of about 1 : 2 from B-ring. Judging from the chemical structures of proanthocyanidin dimers and trimers in *Acacia mearnsii* bark,[18] these ratios would indicate that the extender units consist mainly of profisetinidin and prorobinetinidin types and the terminal unit is a procyanidin type. On the other hand, KBE consists of the procyanidin type as can be seen in most coniferous bark,[19] and Quebracho extracts (QE) consist of the profisetinidin type. From the IC50 value of each ME fraction, which shows almost the same average molecular weights, the inhibitory effect of KBE-ME is the greatest, and those of ABE-ME and QE-ME are similar. Therefore, it is presumed that the hydroxylation patterns of A- and B-rings influence the extent of GTase inhibition.

TABLE 3 Phenyl nucleus composition of several proanthocyanidins analyzed by NER method.

(mol %) *

Sample	A-ring		B-ring		Phl./Res.	Pyr./Cat.
	Phl.	Res.	Cat.	Pyr.		
ABE-ME	15.1	49.5	22.1	38.1	0.31	1.7
-AE	11.3	33.1	17.3	35.6	0.34	2.1
QE-ME	5.3	40.6	64.4	2.0	0.13	0.03
-AE	3.3	43.3	63.6	2.3	0.08	0.04
LBE-EE	9.4	0.6	43.8	2.2	15.6	0.05
-ME	20.5	0.5	65.0	2.8	41.0	0.04
-AE	23.4	0.6	70.3	3.1	39.0	0.04

* These values are represented by mol % to a unit of flavan-3-ol.

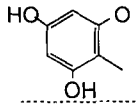
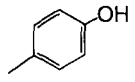
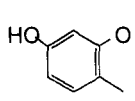
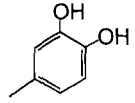
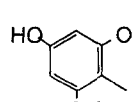
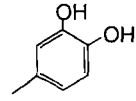
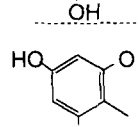
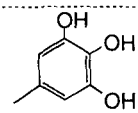
Phl.: phloroglucinol Res.: resorcinol
Cat.: catechol Pyr.: pyrogallol

Sample name: refer to Experimental section.

The relationship between the hydroxylation patterns of non-enzymatically synthesized proanthocyanidins and their GTase inhibitory activities.

Four proanthocyanidins having different hydroxylation patterns were synthesized non-enzymatically, and their inhibitory activities of GTase were examined to understand the relationship between inhibition ability and structure of bark proanthocyanidins. The hydroxylation patterns of the A- and B-rings of these synthesized proanthocyanidins, and their IC_{50} values are shown in Table 4. The AE fraction has a higher inhibitory activity than the ME fraction in all oligomers indicating that the activity depends on their molecular weight in a similar manner to natural polyphenols. PC-ME and PF-ME show the same IC_{50} value of 4.2, which indicates that the hydroxyl group in the A-ring does not influence the inhibitory activity. On the other hand, B-ring hydroxylation patterns resulted in different inhibition levels when comparing PA-ME, PC-ME, and PD-ME. The oligomers containing 3',4'-dihydroxyl groups in the B-ring showed especially high

TABLE 4 The relationship between hydroxylation patterns of non-enzymically synthesized proanthocyanidins and GTase inhibition

Hydroxylation patterns A-ring	Hydroxylation patterns B-ring	Synthesized polyphenols	IC 50 (μg/ml)
		Proapigeninidin-ME	12.7
		-AE	6.2
		Profisetinidin-ME	4.2
		-AE	3.3
		Procyanidin-ME	4.2
		-AE	2.0
		Prodelphinidin-ME	300<
		-AE	75.3

inhibition, whereas oligomers containing 3',4',5'-trihydroxyl groups showed little or no inhibition. These results would explain the reason that Acacia proanthocyanidins having a high proportion of pyrogallol B-ring units showed lower inhibition activity than Karamatsu proanthocyanidins as described above.

Haslam (1974) suggested that the o-dihydroxyl groups of proanthocyanidins are the site of a combination with proteins and also showed that polymeric proanthocyanidins containing many sites for such a combination have a greater ability to precipitate proteins than low molecular weight proanthocyanidins.[20] If this specific association is caused by the hydrogen-bond between polyphenols and proteins, stronger hydrogen-bonds must be formed by pyrogallolic hydroxyl groups than by catecholic hydroxyl groups. However, experimental results were contrary to this expectation. In the formation of polyphenol-protein complexes, hydrophobic interaction in binding sites is regarded as important as hydrogen

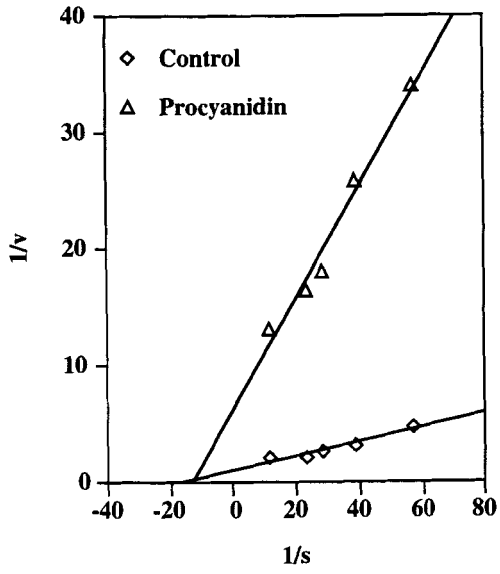


FIGURE 4. Lineweaver-Burk plots of GTase with and without synthesized procyanidin.

bonding. Therefore, the balance of these two types of interactions in the polyphenol molecules may participate in the extent of inhibition of GTase.

The mode of GTase inhibition of synthesized proanthocyanidins

The inhibition mode of the non-enzymatically synthesized procyanidin (PC) oligomer showing high inhibitory effects for GTase was examined from the Lineweaver-Burk plots shown in Fig.4. The K_m value, 5.9 x 10^{-5} M, obtained by addition of the PC oligomer was similar to the control, which indicated that the mode of inhibition was most closely correlated with the classical pattern of non-competitive inhibition in which polyphenol and substrate were assumed to bind simultaneously to the GTase. From the facts presented above, polyphenols would interact with GTase through hydrogen-bonding and hydrophobic interaction at all sites except the reactive center, causing steric changes in the GTase and inhibition of glucan synthesis.

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