Transactions of the Materials Research Society of Japan. Vol. 20

Development of the functional utilization of bark polyphenols

Tohru Mitsunaga, Isao Abe [a]
Masanori Kontani, Hiroyuki Ono and Takaharu Tanaka [b]

[a] Department of Forest Products, Mie University, 1515 Kamihama, Tsu, Mie Pref. 514, JAPAN

[b] Institute for Biomedical Research, Suntory Ltd., Shimamoto, Osaka 618, JAPAN

The chemical modification for wood adhesives and the inhibitory effect for glucosyltransferases (GTase) of bark polyphenols are discussed in this report. The pyran-ring opening of condensed tannins extracted from *Larix* spp bark were observed by the phenolation using boron trifluoride as a catalyst. The modified polyphenols showed higher reactivities with formaldehyde than original bark polyphenols. The inhibition activities of *Larix* and *Acacia mearnsii* bark polyphenols on GTase, which relates intimately with dental caries, were examined. These polyphenols have inhibited GTase strongly, and the effectiveness of *Larix* polyphenols were forty times that of green tea and ten times that of oolong tea polyphenols from their IC_{50} values.

1. INTRODUCTION

While the present-day exhaust mass of bark in Japan reaches 5.6 million tons per year [1], most of this is not utilized and is regarded as troublesome by-products in the lumber industry. However, the bark extracts of some species contain polymeric polyphenols (condensed tannins) mainly, so that to find out the valuable and effective utilization of condensed tannins are very important for the development of forest resources.

The authors have demonstrated that the pyran-ring opened structure is produced by the phenolation of Larix bark extracts in the presence of BF_3, and their reactivity with formaldehyde becomes higher than the original materials.[2][3] They have also elucidated that the bark polyphenols inhibited some enzyme activities strongly. Particularly, it showed a marked inhibition for glucosyltransferases (GTase), which relates intimately with dental caries[4].

This paper reports the results so far obtained in our investigations concerning the structural modification for the new wood adhesives and the GTase inhibition ability for the development of the enzyme inhibitor of bark polyphenols.

2. MATERIALS AND METHODS

2.1. Preparation of bark extracts

Seventy % acetone aq. extracts of acacia (*acacia mearnsii*) bark (ABE) and karamatsu (*Larix* spp.) bark (KBE), and commercially available quebracho (*Schinopsis lorentzii*) extracts (QE) were fractionated by LH-20 gel column chromatography with ethanol, methanol , and 70 % acetone aq. successively. Their eluted fractions were named EE , ME, and AE, respectively.

2.2. Analytical methods of polyphenols

The contents of phenols, flavanols, and tannins for natural polyphenols were determined according to the Folin-Dennis,[5] vanillin-HCl,[6] and Lowenthal methods,[7] respectively. Gel permeation chromatography (GPC) was recorded with a Jasco Trirotar system with Shodex GPC columns KF-802 and KF-804 (4.6mmϕ x 250mm) using

tetrahydrofuran as an eluent.

2.3. Phenolation of bark polyphenols and the reaction with formaldehyde of the products

Dried KBE powder (20 mg) was added to a glass ampule with phenolation reagent (1 ml), phenol : benzene : BF_3-phenol complex = 19 : 10 : 1 v/v, and was heated in a water bath at 40°C. After the phenolation having completed, the reaction mixture was poured into water (200 ml) and then extracted with ethyl acetate (15 ml x 3). The concentrated ethyl acetate solution was added dropwise into benzene (50 ml) to obtain the phenolated products as brownish precipitates. (KBE-P)

About 30 mg of KBE-P was dissolved in 1.5 ml of ethanol containing about 0.3 mmol of formaldehyde. One and half ml of 0.1 N hydrochloric acid was added to the solution. The reaction was carried out at 25 °C in a vial with stirring. The reaction mixture was subjected to column chromatography on a Sephadex LH-20 gel using ethanol as an eluent to remove hydrochloric acid, the remaining formaldehyde, and low molecular weight materials. Adsorbates on the column were eluted with 70 % acetone aq. to give the reaction products. Molecular weights of these products were measured by GPC.

2.4. Synthesis of condensed tannins

Procyanidin (PC), profisetinidin (PF), prodelfinidin (PD), and proapigeninidin (PA) oligomers were synthesized by the condensation of the corresponding flavan-3,4-diols or flavan-4-ol with (+)-catechin under acidic conditions according to the method in our previous paper.[8]

2.5. Preparation of GTase and assay for inhibitory activity

Streptococcus sobrinus 6715 was grown for 16 hr at 37 ° C in 5 l of Todd Hewitt (TH) broth. After the liquid medium having been centrifuged 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. After then, 1 ml of the crude enzyme solution was pipetted into a microtube, and stored in a freezer at -80 °C.

Insoluble glucan synthesized by GTase was measured turbidimetrically with a spectrophotometer 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 polyphenols at 37 °C for 3 hr. The volume of GTase solution used in the assay was determined by that giving absorbance 1.0 at 550 nm. Inhibition rate is expressed by the following equation: Inhibition rate (%) = 100 x (Ac-Ap)/Ac here, Ac and Ap represent absorbance obtained in control and in polyphenol dose, respectively. The IC_{50} means the polyphenol concentration (μg/ml) giving 50 % inhibition of GTase.

3. RESULTS AND DISCUSSION

3.1. The phenolation of bark polyphenols and their reactivity with formaldehyde

In general, resorcinol formaldehyde resins are prepared under acidic or neutral conditions at ambient temperature to form novolak type adhesives. In this study, the condensation of KBE-P with formaldehyde (F) was examined under the conditions of pH 3-4 at 20°C, and the molecular weights of the condensation products of KBE-P with F (KBE-PF) against condensation times are shown in Fig.2. As can be seen, the molecular weight of the control, the reaction product of KBE with formaldehyde (KBE-F), increased slowly. On the other hand, that in the KBE-PF increased rapidly and reached three times the molecular weight compared with prior to the condensation after 24 hours. The molecular weight of KBE-PF decreased with increases of the treatment time in the phenolation because the amount of phloroglucinolic nuclei remaining in the A-ring decreased during the

phenolation. Takano has mentioned that the molecular weight reduction of polyphenols was useful in the production of cold-setting adhesives.[9] Therefore, the phenolation of condensed tannins in the presence of BF_3 seems to be a suitable technique for decreasing the molecular weights, and additionally, it also increases the rate of reactions with F by their pyran-ring openings.

3.2. Inhibitory activity of bark polyphenols

Green tea and oolong tea are known as the preventive beverages for dental caries, and the inhibition of GTase on polyphenols contained in these beverages have been examined.[10] Table 1 shows the contents of phenols, flavanols, and tannins of several polyphenol materials Flavanols contents of GTE and OTE are very similar, but their tannins contents are quite different. Because GTE contains mainly monomeric polyphenols, in which it does not have tanning characteristics. The tannins contents of ABE and KBE occupy about 80% of flavanol contents, which indicates that bark extracts consist mainly of the oligomeric polyphenols because it is known that the polyphenol consisting of molecular size beyond flavan-3-ol dimer show extreme tanning properties.[11] The relationship between the concentration of these extracts

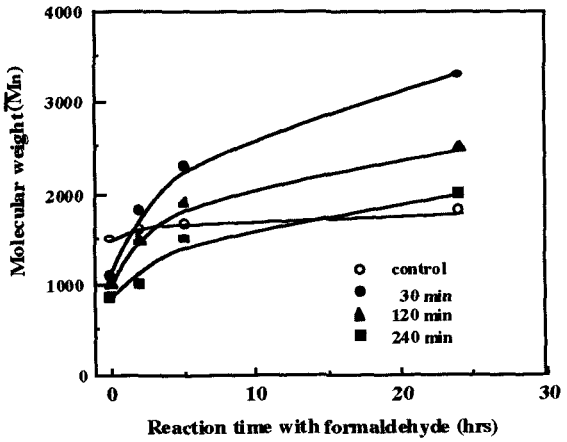


Fig. 1 **Changes of molecular weight of the products in the reaction of phenolated karamatsu bark extracts with formaldehyde.**

Note: Times in figure represent the treatment time of phenolation. Control is 70 % acetone aq. extracts of karamatsu.

Table 1 Polyphenol analyses of teas and barks extracts by 70% acetone aq.

Extracts	Phenols	Flavanols	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

Phenol, flavanol, and tannin contents (%) on (+)-catechin were measured by Folin-Dennin, vanillin-HCl, and Lowenthal method, respectively.

Sample name: refer to experimental section

and their inhibition rate on GTase is shown in Fig. 3. Both bark extracts exhibited higher inhibition rates than GTE or OTE. Particularly KBE showed very high inhibition rate in spite of crude products, and its IC_{50} value was about 2.0 (μg/ml). Judging from these IC_{50} values, inhibitory effect of KBE was about ten times as high as that of OTE and forty times of GTE. Therefore, GTase inhibitory activity of these extracts was presumed to relate to the contents and structures of polyphenols.

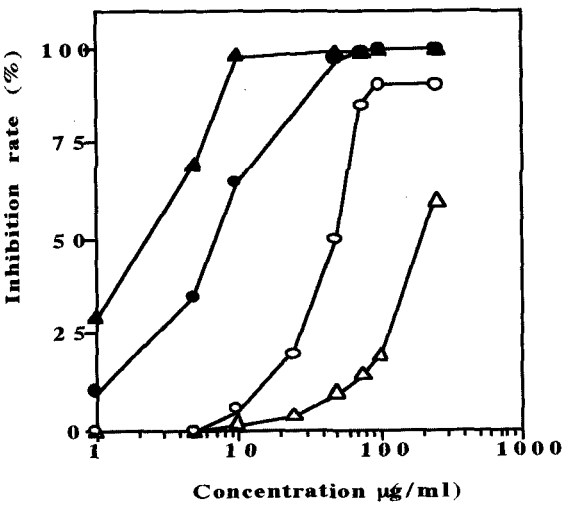


Fig. 2 **GTase inhibitory activity of 70 % acetone extracts from teas and bark**

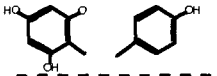
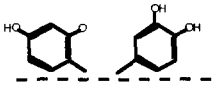
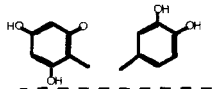
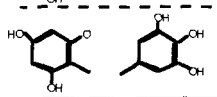
Legend : ▲ KBE ● ABE ○ OTE △ GTE

Sample name: refer to experimental section

3.3. The relationship between the hydroxylation patterns and GTase inhibitory activities of synthesized condensed tannins

Four condensed tannins having different hydroxylation patterns were synthesized, and their inhibitory activities of GTase were examined to understand the relationship between the inhibition ability and structure of natural polyphenols. The hydroxylation patterns in A- and B-rings of these synthesized condensed tannins, and their IC_{50} values are shown in Table 2. AE fraction has higher inhibitory activity than ME fraction in all oligomers, so that, the activity depends on their molecular weight in a similar manner as natural polyphenols. PC-ME and PF-ME show the same IC_{50} values of 4.2, which indicates that the hydroxyl group in A-ring does not influence the inhibitory effects. On the other hand, B-ring hydroxylation patterns give the different inhibition rates, in comparison of PA-ME, PC-ME, and PD-ME. The oligomers containing catecholic hydroxylation pattern showed high inhibition abilities especially, but that containing pyrogallolic one, which was expected to have a strong hydrogen bond to enzyme, had little or no effect on the inhibition. These results explain the reason that acacia polyphenols having pyrogallolic hydroxyl group in some B-ring units showed lower inhibition activity than karamatsu polyphenols. Haslam suggested that the o-dihydroxyl groups of proanthocyanidins are the site of combination with proteins and showed that polymeric polyphenols having many sites for such a combination have a greater ability to precipitate proteins than the polyphenols being low molecular weight.[12] If this specific association is caused by the hydrogen-bond between polyphenols and proteins, a stronger hydrogen-bond should be formed by pyrogallolic hydroxyl groups than by catecholic ones. However, the expected idea on GTase inhibition opposed the experimental results. In the formation of polyphenol-protein complex, hydrophobic interaction in binding

Table 2 The relationship between hydroxylation patterns of synthesized polyphenols and the GTase inhibition

Hydroxylation patterns A-ring	Hydroxylation patterns B-ring	Synthesized polyphenols	IC_{50} (μg/ml)
		PA-ME	12.7
		-AE	6.2
		PF-ME	4.2
		-AE	3.3
		PC-ME	4.2
		-AE	2.0
		PD-ME	300<
		-AE	75.3

sites is regarded as important as hydrogen bonding. Therefore, the balance of these two types of interactions in the polyphenols molecules may participate in the extent of inhibition on GTase.

REFERENCES

1. M. Matsuyama, Mokuzai Kougyou, 38, (1983) 492
2. T. Mitsunaga, I. Abe, Mokuzai Gakkaishi, 38, (1992) 585
3. T. Mitsunaga, O. Kondo, I. Abe, Mokuzai Gakkaishi 41, (1995) 193
4. T. Mitsunaga, Abstract of The Japan Wood Research Society, Tokyo, (1995) 647
5. T. Swain and W. E. Hillis, *J. Sci. Food Agric.* 10, (1959) 63.
6. R. B. Broadhurst and W. T. Jones, *J. Sci. Food Agric.*, 29 (1978) 788.
7. M. Samejima and T. Yoshimoto, *Mokuzai Gakkaishi*, 27, (1981) 491.
8. T. Mitsunaga, A. Yoshida, I. Abe, Mokuzai Gakkaishi, 41, (1995) 193
9. R. Takano, *J. The Toyama Forestry and Forest Prod. Res. Center*, 6, (1992) 50
10. K. Nakahara, et al., *Applied and Envionmental Microbiology*, (1993) 968
11. H. Kawamoto,et al., *Mokuzai Gakkaishi*, 37, (1991) 741
12. E.Haslam *Biochem. J.* 139, (1974) 285