J Wood Sci (2003) 49:461–465
DOI 10.1007/s10086-002-0496-4

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Tyrosinase inhibitory activity of proanthocyanidins from woody plants

Received: January 24, 2002 / Accepted: October 11, 2002

Abstract Flavanol values, molecular weight distributions, polyphenolic patterns, and tyrosinase inhibitions of proanthocyanidins (PACs) from karamatsu (*Larix* sp.) bark, acacia Morishima (*Acacia mearnsii*) bark, and commercial quebracho (*Schinopsis lorentzii*) extracts were examined to investigate the relation between the structures of PACs from woody plants and tyrosinase inhibitory activities. Aqueous acetone (70%) extracts of acacia showed high flavanol contents and strong tyrosinase inhibition. On the other hand, the quebracho extracts inhibited tyrosinase activity slightly despite its high flavanol content. Phenyl nucleus analysis by the nucleus exchange reaction provided information that karamatsu, quebracho, and acacia extracts consist of procyanidin, profisetinidin, and prorobinetinidin, respectively. The relation between the phenolic hydroxylation pattern and tyrosinase inhibition suggested that the PACs with a 5,7-dihydroxyphenyl structure in the A-ring and a 3,4,5-trihydroxyphenyl structure in the B-ring have potent tyrosinase inhibitory activity.

Key words Tyrosinase inhibition · Proanthocyanidin · Condensed tannin · Nucleus exchange reaction

Introduction

Proanthocyanidin, commonly termed condensed tannin, which is widely distributed over the plant kingdom, is found in many tissues that protect living bodies, especially bark,[1–3] seed coat,[4] and fruit skin.[5] It is considered to be a defensive component against other living things. At present, the protein precipitation ability or enzyme inhibitory activity of proanthocyanidins (PACs) could be explained as protective functions. Additionally, some reports showed utilization of these characteristics, such as inhibition of glucosyltransferase[6,7] related to dental caries, inhibition of angiotensin I-converting enzyme[8] related to hypertension in humans, and antitermite activity.[9]

We have found that PACs contained in the pericarp of Jatoba (*Hymenaea courbaril*), used as a herbal medicine in Brazil, inhibits tyrosinase activity.[10] Tyrosinase is an enzyme that catalyzes the biosynthesis of melanin pigment in melanocytes of human skin from tyrosine or dihydroxyphenylalanine (DOPA) as the substrate. Therefore, substances that inhibit tyrosinase activity are expected to be whitening agents that have application to pharmaceuticals and cosmetics. There have been some recent reports on wood extractives relating to tyrosinase inhibitory active compounds such as chlorophorin from *Artocarpus incisus*,[11] β-thujaplicin from *Cupressus lusitanica*,[12] and sequirin-C from *Cryptomeria japonica*.[13] Tyrosinase inhibition of PACs from woody plants has never been investigated. Therefore, this report discusses the influence of structural features of PACs from bark and wood (i.e., flavanol contents, molecular weight distributions, phenolic hydroxylation patterns) on tyrosinase inhibitory activities.

Materials and methods

Preparation of samples

Dried karamatsu (*Larix* sp.) bark, acacia Morishima (*Acacia mearnsii*) bark, and commercially available quebracho (*Schinopsis lorentzii*) heartwood hot water extracts were extracted with 70% aqueous acetone. These extracts were filtered and evaporated in vacuo. The residue was then lyophilized to give the crude extractives (AWE). The AWE was applied to a Sephadex LH-20 (Amersham Pharmacia, Uppsala, Sweden) column (35 mm i.d. × 45 cm) and eluted

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Part of this paper was presented at the 49th Annual Meeting of the Japan Wood Research Society, Tokyo, April 1999

with water (500 ml), ethanol (500 ml), methanol (500 ml), and 70% aqueous acetone (500 ml) successively. Each fraction was evaporated and lyophilized to give amorphous powder, represented as WE, EE, ME, and AE, respectively.

Assay for tyrosinase inhibitory activity using L-tyrosine as substrate

Tyrosinase inhibitory activity was measured according to the method described in our previous paper.[10] That is, 1.0 ml of 1.66 mM L-tyrosine (Wako Pure Chemical Industries, Osaka, Japan) solution and 1.0 ml of the 1.0 mg/ml test sample in 10% (v/v) aqueous dimethylsulfoxide (DMSO) solution were mixed with 0.9 ml of 30 mM sodium phosphate buffer (pH 6.8) and preincubated at 37°C for 5 min. Finally, 0.1 ml of the tyrosinase (EC 1.14.18.1; Sigma Chemical, St. Louis, MO, USA) solution (900 units) was added. This mixture was incubated at 37°C for 10 min, and the absorbance was measured at 475 nm. Tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = [(A) - (B - C)]/(A) \times 100$$

where A represents the absorbance of the solution in the absence of the test sample and the presence of the enzyme after incubation; B represents the absorbance of the solution in the presence of the test sample and the enzyme after incubation; and C represents the absorbance of the solution in the presence of the test sample and the absence of the enzyme after incubation. Each experiment was carried out in triplicate.

Assay for tyrosinase inhibitory activity using L-3,4-dihydroxyphenylalanine as substrate

Tyrosinase inhibitory activity was measured according to the method described in our previous paper.[10] That is, 10 mM sodium phosphate buffer (pH 6.8) containing 0.55 mM L-3,4-dihydroxyphenylalanine (L-DOPA) (Wako) was preincubated at 25°C for 10 min. Then 2.8 ml of this solution was mixed with 0.1 ml of the 10.0 mg/ml test sample in DMSO. Finally, 0.1 ml of the tyrosinase solution (900 units) was added to the mixture and incubated at 25°C. The absorbance of the mixture was measured at 475 nm between the incubation times of 1.75 and 2.75 min. Tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = (A - B)/(A) \times 100$$

where A represents the difference in the absorbance of the solution in the absence of the test sample and the presence of the enzyme; and B represents the difference in the absorbance of the solution in the presence of the test sample and the enzyme. Each experiment was carried out in triplicate.

Vanillin-hydrochloric acid method

The flavanol contents were estimated by the vanillin-hydrochloric acid method.[14] That is, 1 ml of the 0.8 mg/ml methanolic solution was placed in a 25-ml brown test tube to which 6 ml of methanol solution containing 4% (w/v) vanillin was added. After stirring vigorously, 3 ml of concentrated hydrochloric acid was added to the solution. After stirring with a tube mixer every 5 min for 15 min, the absorbance was measured by a Jasco V-520 UV/VIS spectrophotometer. The absorbance for karamatsu extracts was recorded at 500 nm, and those for the acacia and quebracho extracts at 540 nm. The amount of flavanol was calculated from the calibration curve created on the basis of (+)-catechin absorbance at 500 and 540 nm.

Gel permeation chromatography

Samples (2–3 mg) were dissolved in 1 ml of rectified tetrahydrofuran (THF), and molecular weight distributions were obtained by gel permeation chromatography (GPC)[15] with a Jasco Trirotar system with Shodex GPC columns KF-804 and KF-802 (4.6 mm i.d. × 25 cm) (Showa Denko, Tokyo, Japan) using THF as eluent. The flow rate was 1 ml/min, and the temperature was 25°C. The absorbance of the eluate was monitored at 280 nm. The THF-insoluble sample was made into an acetylated substance and similarly measured. Acetylation was executed as follows: After addition of 500 μl of pyridine and of 500 μl of acetic anhydride in 10 mg of the sample, the mixture was allowed to stand in the dark overnight. It was then dropped in excess cold water and extracted three times by 30 ml of chloroform using a separatory funnel. The chloroform layer was washed with 30 ml of 5% sodium hydrogen carbonate solution, 30 ml of 5% hydrochloric acid solution, and 30 ml of water, respectively, The chloroform solution was dried overnight by anhydrous sodium sulfate. After filtering, the filtrate was evaporated in vacuo and subsequently lyophilized to give the acetylated substance. The chromatogram was calibrated using (+)-catechin, synthesized procyanidin dimer, and standard polystyrenes (molecular weights 2200, 9000, and 25 000 daltons, respectively). The number average molecular weight and the weight average molecular weight ($\overline{M}_n$, $\overline{M}_w$) were calculated from the molecular weight distributions using a Jasco 807-IT integrator.

Analysis of PAC by the nucleus exchange reaction

The nucleus exchange reaction (NER)[16] was carried out in a glass ampul with 15 mg of the sample and 1 ml of nucleus exchange reagent [benzene/phenol/BF_3–phenol complex salt at 10:19:3 (v/v)]. The ampul was heat-sealed and the reaction took place for 4 h at 80°C or 150°C to estimate the free phenol nucleus of the A-ring or the B-ring, respectively. After the reaction the ampul was cooled to room temperature, and the reaction mixture was transferred quantitatively to a separatory funnel with 50 ml of saturated sodium chloride aqueous solutions to render the BF_3 inactive. After extraction by 20 ml of ethyl acetate three times, 0.6 mg of dibenzyl in benzene was added as internal standard to the ethyl acetate layer; then sodium sulfate anhydrous was added, and the solution was left to cool in the dark overnight. Insoluble materials were filtered and

Table 1. Tyrosinase inhibition, flavanol values, and molecular weight distribution of fractions separated by LH-20 from 70% aqueous acetone extract

Sample[a]	Tyrosinase inhibition (%)		Flavanol value (%)	$\overline{M_n}$	$\overline{M_w}$	d	DP
	Tyrosine	DOPA					
K-EE	23.3	34.4	16.5	325	750	2.3	2.2
K-ME	32.3	51.5	42.6	1150	1585	1.4	4.0
K-AE	34.0	42.9	46.0	2852	4825	1.7	13.5
A-EE	57.0	65.0	30.1	389	758	1.9	1.6
A-ME	83.1	84.7	59.5	1045	1495	1.4	3.9
A-AE	79.2	83.4	59.5	2474	4235	1.7	11.4
Q-EE	47.1	69.2	74.4	367	1834	5.0	1.9
Q-ME	3.4	17.8	80.4	902	1464	1.6	3.9
Q-AE	11.0	31.7	46.5	948	2285	2.4	8.0

[a] K, karamatsu (*Larix* sp.); A, acacia morishima (*Acacia mearnsii*); Q, quebracho (*Schinopsis lorentzii*). Fractions EE, ME, and AE were fractionated by a LH-20 column eluted with ethanol, methanol, and 70% aqueous acetone, successively, from 70% aqueous acetone extracts DOPA, dihydroxyphenylalanine; d, dispersity ($\overline{M_w}/\overline{M_n}$), where $\overline{M_w}$ is the weight average molecular weight, and $\overline{M_n}$ is the number average molecular weight; DP, degree of polymerization of top peak of gel permeation chromatography (GPC)

washed with ethyl acetate. The filtrate and washings were combined and evaporated to a small volume.

A 100-μl portion of this solution was transferred to a small vial to which the same volume of *N,O*-bis(trimethylsilyl)acetamide (BSA) was added. After a 1-h derivatization reaction at room temperature, the trimethylsilane (TMS) derivatives were analyzed quantitatively using gas-liquid chromatography (GLC). GLC was performed by a Yanagimoto G-180 with a hydrogen flame ionization detector (FID) using a Quadrex S2005 (0.25 mm i.d. $\times$ 25 m; film thickness 0.25 μm) methyl silicone capillary column. The column temperature of the GLC was maintained at 130°C for 1 min and then increased to the final temperature of 190°C at a rate of 3°C/min. The injector and the detector temperatures were both 230°C, and the carrier gas used was He (1.2 kg/cm^2).

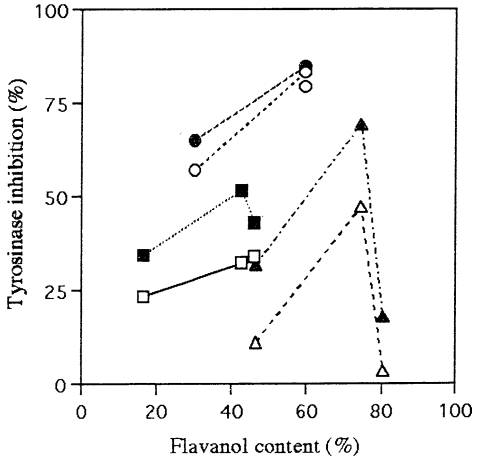

Fig. 1. Relation between flavanol contents and tyrosinase inhibition. *Squares*, karamatsu extracts; *circles*, acacia extracts; *triangles*, quebracho extracts. *Open symbols*, tyrosine was used as a substrate; *closed symbols*, dihydroxyphenylalanine (DOPA) was used as a substrate

Results and discussion

Relation between flavanol contents and tyrosinase inhibition

The relations of the flavanol contents and tyrosinase inhibitions of the fractions separated by LH-20 column chromatography of 70% aqueous acetone extracts (AWE) are shown in Table 1 and Fig. 1. The flavanol contents in the methanol fraction (ME) and 70% aqueous acetone fraction (AE) were more than twice that of the ethanol fraction (EE) in karamatsu and acacia extracts; stronger tyrosinase inhibition was observed in the ME and AE fractions than in the EE fraction. Moreover, the acacia extracts showed higher flavanol content and stronger tyrosinase inhibition than the karamatsu extracts. Nevertheless, the ME fraction in the quebracho extracts showed the highest flavanol value of all the fractions, containing more than 80%; tyrosinase inhibition of the fraction was hardly observed. Although it was presumed that the higher flavanol content in the extractives the stronger the tyrosinase inhibition observed, a clear relation between the flavanol content and tyrosinase inhibition was not present. According to the report on tyrosinase inhibition of flavonoids,[17] the substituted position of the hydroxyl group on the phenolic ring was greatly affected by tyrosinase inhibition, rather than by the flavanol content in the extracts. Therefore, the structural features of PAC, the degree of polymerization, and the hydroxylation pattern on the aromatic ring were investigated with respect to tyrosinase inhibition.

Tyrosinase catalyzes hydroxylation of L-tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone. Two substrates were used in this experiment to investigate differences in the catalytic activities in two reactions (hydroxylation and oxidation). The specificity of the inhibitory activity

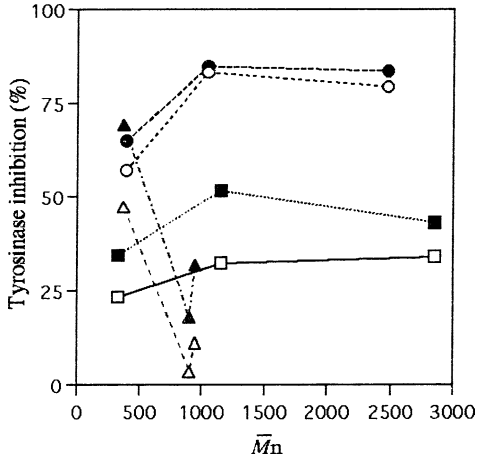

Fig. 2. Relation between average molecular weight and tyrosinase inhibition. *Squares*, karamatsu extracts; *circles*, acacia extracts; *triangles*, quebracho extracts. *Open symbols*, tyrosine was used as a substrate; *closed symbols*, DOPA was used as a substrate

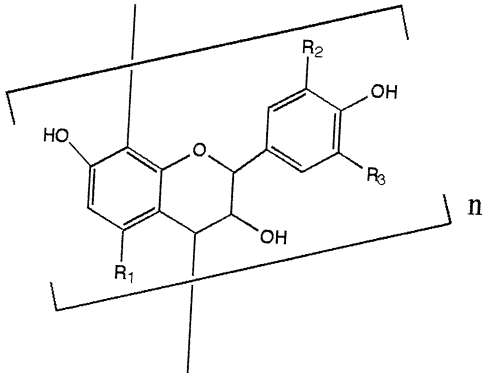

Fig. 3. Chemical structure of proanthocyanidins. Procyanidin, R_1=OH, R_2=OH, R_3=H; profisetinidin, R_1=H, R_2=OH, R_3=H; prorobinetinidin, R_1=H, R_2=OH, R_3=OH

was not observed, but these PACs inhibited the oxidation of DOPA to dopaquinone rather than the hydroxylation of tyrosine to DOPA.

Relation between the degree of polymerization of PAC and tyrosinase inhibition

The relations of molecular weight distributions and tyrosinase inhibitions of the fractions separated by LH-20 column chromatography of AWE are shown in Table 1 and Fig. 2. The molecular weights of these fractions in any wood species increased in the following order: EE, ME, and AE. It has already become clear that the ME fractions of karamatsu and acacia contained the same amounts of flavanols as the AE fractions of each wood species, and that the degree of flavanol polymerization of the AE fractions of these species was three times higher than that of the ME fractions. The high-molecular-weight fraction of PAC is thought to have high protein precipitation ability[18] and potent inhibitory activity against some enzymes.[19] Therefore, the AE fraction was expected to show stronger tyrosinase inhibition than the ME fraction, but that relation was not observed between the degree of polymerization of PACs and tyrosinase inhibition in the PACs from karamatsu and acacia. On the other hand, the effect of bark PAC on glucosyltransferase (GTase) inhibition was reported to inhibit the AE fractions of both karamatsu and acacia more strongly than the ME fractions.[6] Thus, the mechanism in tyrosinase inhibition of PACs was thought to be different from that of GTase inhibition. The flavanol structures of PACs in the ME and AE fractions might influence tyrosinase inhibition because the C_6-C_3 structure, existing in the C-ring through the B-ring of the flavanol unit, is similar to that in the substrates of tyrosinase.

Table 2. Phenyl nucleus analysis of proanthocyanidins in the fractions separated by LH-20 from 70% aqueous acetone extract

Sample[a]	A-ring		B-ring	
	Phloroglucinol	Resorcinol	Pyrogallol	Catechol
K-ME	20.5	0.5	2.8	65.0
K-AE	23.4	0.6	3.1	70.3
A-ME	15.1	49.5	38.1	22.1
A-AE	11.3	33.1	35.6	17.3
Q-ME	5.3	40.6	2.0	64.4
Q-AE	3.3	43.3	2.2	63.6

The values are the mol% to a unit of flavan-3-ol
[a] Fractions ME and AE were fractionated by a LH-20 column eluted with methanol and 70% aqueous acetone, successively, from 70% aqueous acetone extracts

Influence of the phenyl nucleus constituted PAC on tyrosinase inhibition

The NER is an analytical method designed to solve the complicated structures of lignin[20] and condensed tannin.[16] This method is available to determine if the phenyl nuclei constitute lignin or condensed tannin. For instance, phloroglucinol and catechol are liberated from the procyanidin type of PAC, and resorcinol and catechol are liberated from the profisetinidin type. The data obtained by NER of fractions obtained from AWE are shown in Table 2, and the structures of PACs in woody plant extracts are shown in Fig. 3. The ME and AE fractions of karamatsu consist of procyanidin-type PACs and those of quebracho consist of the profisetinidin type. Moreover, the ME and AE fractions of acacia liberate phloroglucinol and resorcinol from the A-ring and pyrogallol and catechol from the B-ring. Thus, acacia extracts consist of mixed PACs of prorobinetinidin as the major unit and profisetinidin as the minor unit.

Prorobinetinidin-type PACs from *Acacia mearnsii* bark[9] and the procyanidin-type from *Larix leptolepis* bark[21] have been reported, and the results support our NER analytical data. Karamatsu extracts and quebracho extracts inhibited tyrosinase activities slightly, and procyanidin composed of the phloroglucinolic moiety in the A-ring showed stronger

tyrosinase inhibition than profisetinidin composed of the resorcinolic moiety in the A-ring. Although acacia extracts contained profisetinidin, it showed the strongest tyrosinase inhibition of the three kinds of woody plant extracts. Gallic acid and its derivatives were reported to serve not only as tyrosinase inhibitors but also as tyrosinase substrates and show strong tyrosinase inhibition.[22] As acacia extracts consist of mainly prorobinetinidin composed of the pyrogallolic moiety in the B-ring, the prorobinetinidin of acacia extracts was thought to have greater tyrosinase inhibitory activity than profisetinidin (composed of the catecholic moiety in the B-ring). Therefore, the hydroxylation pattern of the B-ring was thought to contribute to tyrosinase inhibition more so than that of the A-ring, and the pyrogallolic and phloroglucinolic moieties in PACs may influence tyrosinase inhibition. As a result, tyrosinase inhibitory activity was closely related to the phenolic hydroxylation pattern of PACs. However, it is necessary to investigate the structure–activity relation in detail using synthesized PACs consisting of several polyphenolic patterns.

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