The Chemical Structure of Kaki-tannin from Immature Fruit of the Persimmon (Diospyros kaki L.)

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Received November 28, 1977

A partially purified tannin was prepared by a K$_2$HPO$_4$-precipitation method from immature persimmon fruit (Diospyros kaki L., cv. "Hiratanenashi," astringent type). The kaki-tannin gave delphinidin and cyanidin on acid hydrolysis. Its methylated derivative showed $\nu_{\text{max}}$ 1720 cm$^{-1}$ in IR spectrum and had the molecular weight of ca. $1.38 \times 10^4$ daltons in MW. From the results of toluene-$\alpha$-thiol treatment, it is suggested that the kaki-tannin consists of catechin, catechin-3-gallate, gallocatechin, gallocatechin-3-gallate and an unknown terminal residue, and belongs to proanthocyanidin B group with a carbon-carbon interflavan linkage from C-4 of one unit to C-6 or C-8 of another unit.

An immature fruit of persimmon (Diospyros kaki L.) has remarkable astringency. It is due to water-soluble tannin(s) present in the tannin cells of the fruit. The tannin(s), called "kaki-tannin" here, has strong protein-binding capacity and is positive in the ferric chloride test showing indigo coloration. In Japan, umbrellas were used to be made from the paper which was soaked into the crude kaki-tannin extract and fishing nets from the fiber were also similarly treated. Now it has been widely used as a de-proteining agent in the brewing process of sake, Japanese rice wine. These various kinds of utilization of kaki-tannin have excited our intense interest in its chemical structure.

The first chemical study of kaki-tannin was initiated by Komatsu and Matsunami in 1923. They reported that kaki-tannin had an elemental formula of C$_{14}$H$_{20}$O$_9$, and contained gallic acid and phloroglucin in the molecule. Their kaki-tannin preparation, however, appeared to contain much impurity. In 1962, Ito and Oshima proposed leucodelphinidin-3-glucoside for the major component of kaki-tannin from the following experimental evidence: i) Treatment with stronger acid yielded delphinidin and glucose. ii) Alkali degradation of methylated kaki-tannin preparation produced dimethyl phloroglucin and trimethyl gallic acid. Further investigation by Ito and Joslyn revealed that the structure of kaki-tannin might be more complex. Thus, when heated with dilute hydrochloric acid kaki-tannin readily yielded gallic acid, gallocatechin, and gallocatechin gallate. They suggested from these evidence that kaki-tannin was a kind of conjugated tannin; the major component was leucodelphinidin, to which gallic acid, gallocatechin, and gallocatechin gallate conjugated in this molecule.

However, there are still many open questions on the chemical structure of kaki-tannin. Kaki-tannin has following properties: i) it easily gelates at high concentrations, ii) it gives mainly delphinidin with a small amount of cyanidin on strong acid hydrolysis, iii) it is not or hardly soluble in n-butanol, isopropanol, acetone, and ethyl acetate, iv) it elutes at a void volume in Sephadex LH-20 column chromatography with the solvent of 70% aqueous dimethyl sulfoxide, v) it may have about twice as many phenolic hydroxyl groups per A ring as catechin, estimated by vanillin/Folin-Denis coefficient. All these reported results may not be in complete accordance with the chemical structure of kaki-tannin proposed by them. In addition, there
are no clear chromatographical analysis and precise estimation of the molecular weight of kaki-tannin in all reports. It has been generally said that kaki-tannin is a kind of conjugated tannin or condensed tannin, but these terms are arbitrarily used without clear chemical definition.

We tried to isolate purified kaki-tannin under a mild condition from the methanol extract of immature persimmon fruit, using the ferric chloride test for detection. After examining various procedures we found the K$_2$HPO$_4$-precipitation procedure was most excellent for the purification of kaki-tannin which gave kaki-tannin preparation of 1.3~1.6% on fresh weight basis. This value was nearly coincident with that determined by the micro-Lowenthal method. The IR spectrum of this preparation is almost identical to that of polyethylene glycol-precipitated tannin, particularly, in the absorption pattern at about 1700 cm$^{-1}$ which is attributable to carbonyl groups, e.g., ester linkages. There is no or less observation of IR absorption caused by polyethylene glycol in the IR spectrum of polyethylene glycol-precipitated tannin. This indicates that this kaki-tannin is hardly degraded during the preparation involving acidic treatment. The fact that the methylated kaki-tannin showed $\nu_{max}$ 1720 cm$^{-1}$ in the IR spectrum suggested that it has ester linkages in the molecule. The absorption was obscure in the IR spectrum of non-treated kaki-tannin, $\nu_{max}$ 1690 cm$^{-1}$ and more dull. Our preparation of kaki-tannin seems to mainly consist of highly polymerized or condensed polyphenols, since on cellulose-TLC with two dimensional development in the solvents of (C) and (D) no spot of polyphenols was observed, except at or near the origin. It gave anthocyanin-like pigments on acid treatment. When heated in 2 N hydrochloric acid or sulfuric acid, it not only decomposed to liberate delphinidin and some cyanidin but also progressively polymerized to yield amorphous and brown phlobaphen-like substances which precipitated during acid hydrolysis. The quantitative ratio of cyanidin to delphinidin was approximately 1 to 3 when estimated by the spot area on cellulose-TLC. In addition, various kinds of polyphenol components were detected in the acid-hydrolyzate on the cellulose-TLC with two dimensional development. Apparently it is difficult to study the structure of kaki-tannin only by acid hydrolysis, since polyphenols condense easily with each other under the acidic condition.

Thompson et al.$^{10}$ reported in details that toluene-$\alpha$-thiol was an excellent reagent for examining the structure of procyanidin B groups because it cleaved trimeric and dimeric proanthocyanidins at their carbon-carbon linkages, and free flavan-3-ol from the lower unit and the thiol derivative from the upper unit were released in ethanolic acetic acid. No polyphenol was released from kaki-tannin with the acidic ethanol incubation without toluene-$\alpha$-thiol for 4 hr-reflux. However, 3 hr after the addition of toluene-$\alpha$-thiol to the same mixture on the two dimensional cellulose-TLC some spots of polyphenols were detected. The size of their spots increased with incubation time. They were reanalyzed by silica gel-TLC with the solvents of (E) and (F), and the chromatogram gave four obvious spots which were positive to bis-diazotized benzidine reagent and hydrochloric acid-vanillin reagent. The degraded products were termed F-1, F-2, F-3, and F-4 in the order of the $Rf$ value, from high to low, obtained with the solvent of (E). The quantitative ratio of the four degraded products by toluene-$\alpha$-thiol treatment was examined with regard to incubation time (0.5, 1, 2, 4, 24, and 55 hr), using the coloration of the spots with hydrochloric acid-vanillin reagent which reacts with phloroglucinol groups, in this case the A ring of the products. After half an hour incubation a large amount of polyphenols were observed to remain still at the origin of silica gel-TLC, but noticeably these four products were already produced in such a short time. The ratio of F-1 to F-3 was 1 to 1.8 and the amount of F-2 produced and particularly that of F-4 was little in comparison with other products by the estimation of the spot area on silica gel-TLC. After 1 hr incubation the ratios of F-1, F-2, F-3, and F-4 changed to 1, 0.8, 2, and 1,
The ratio finally reached constant values of 1, 1, 2, and 2, respectively, in the samples of 4, 24, and 55 hr incubation. While the ratios remained the same even after 24 and 55 hr, the amount of the spots at the origin of silica gel-TLC decreased gradually with incubation time. When the final residue was analyzed, the kaki-tannin preparation was found to contain a small amount of inorganic salts and a trace of carbohydrates in addition to the polyphenol components.

These findings demonstrate that kaki-tannin is chiefly composed of these four components with a quantitative ratio of 1, 1, 2, 2, and has some partial structure which tends to produce more rapidly F-1 and F-3 with the ratio of 1 to 2 than other two components in a short incubation.

In the following experiments, the four degraded products were isolated by silica gel partition chromatography and each of them showed a single spot on silica gel-TLC with two kinds of solvent, (E) and (F). By tannase treatment F-2 gave gallic acid and F-1, whereas F-4 did gallic acid and F-3. The presence of gallic acid ester linkage in both molecules was confirmed by the observation of $\nu_{max}$ 1720 cm$^{-1}$ in the IR spectra of methylated F-2 and F-4 by means of diazomethane.

After methylation each of them was found to have a molecular ion of 468, 662, 498, and 692, respectively, by mass spectrometry. The mass spectrum of methylated F-1 was similar to that of methylated F-3 in a higher mass range with a shift of 30 mass unit. A similar shift of 30 mass unit was also recognized between methylated F-2 and methylated F-4. Examination of each fragmented peak offered the mass fragment pattern as shown in Fig. 1. From these experimental evidence the structures of these four degraded products from kaki-tannin were formulated as shown in Fig. 2. They are the thioether of catechin, catechin-3-gallate, gallocatechin, and gallocatechin-3-gallate.

The structure was supported obtained by the $^1$H-NMR spectrum in (CD$_3$)$_2$CO with or without D$_2$O. The overall proton integration of these products were in agreement with the above molecular formulas. The NMR spectrum of F-3 in (CD$_3$)$_2$CO with D$_2$O was very similar to that of (2R,3S,4S)-4-benzylthioflavan-3,3',4',5,7-pentaol reported by Thomp-son et al.,$^{10}$ except the presence of hydroxyl group at the C-5' of the B nucleus of the flavan-3-ol derivative. The result appears to indicate that kaki-tannin consists of (-)-epicatechin and (-)-epigallocatechin. How-

FIG. 1. Mass Fragmentation Patterns of Methylated F-2 and F-4.
FIG. 2. Presumed Structures for F-1, F-2, F-3 and F-4.

ever, Ito and Joslyn6) demonstrated that their persimmon tannin preparation yielded (−)-gallocatechin and (−)-gallocatechin gallate by weakly acidic treatment (0.1 N HCl, 20 min in boiling water) using two dimensional paper chromatography. The stereochemistry of the four polyphenol components is now under detailed investigation. The NMR spectrum of F-4 (or F-2) showed three significant differences from that of F-3 (or F-1): an additional two proton resonance (7.04 ppm, singlet) due to the aromatic protons of the galloyl group, a downfield displacement of the proton absorption at C-3 (4.00 ppm, multiplet to 5.52 ppm, doublet) as a result of acylation of the 3-hydroxyl group, and the alteration of 5.93 (1H, doublet) and 6.06 (1H, doublet) ppm to 6.06 ppm (2H, singlet) in 2 protons existing in A ring (C-6 and C-8).

There has been no precise data on the molecular weight and its distribution of kaki-tannin. Generally, kaki-tannin is said to be a high molecular weight compound. The difficulty in measuring the molecular weight of kaki-tannin seems to be caused by the strong hydrogen bond-forming ability of this molecule, since kaki-tannin aggregates each other, easily gelates, and firmly adsorbs to various adsorbents and other materials in various chromatographies because of a surprisingly large number of phenolic hydroxyl groups present.

Therefore, we tried to perform gel permeation chromatography of methylated kaki-tannin, masking phenolic hydroxyl groups. Considering the degradation of kaki-tannin during the methylation process and the cleavage of hydrogen bonds in the solvent, kaki-tannin was treated with four kinds of the methylation procedure: i) by diazomethane in methanol, ii) by dimethyl sulfate in 50% KOH in an ice bath, iii) by the Kuhn method, Ag₂O + CH₃I in dimethyl sulfoxide, iv) by a modified-Hakomori method,¹¹ NaH + CH₃I in dimethyl sulfoxide. After extraction with chloroform kaki-tannin methylated by these methods were respectively analyzed by high speed liquid chromatography.

All of these methylated preparation showed one major peak with a few shoulders in the chromatograms. When methylated by diazomethane, an extremely high molecular weight species was observed, having a major peak of ca. 2.5 × 10⁴ and a minor peak of ca. 8.0 × 10⁴ daltons. On the other hand, the molecular weight distribution of the other three methylated preparations were in a range of ca. 1.38 to 2.5 × 10⁴ daltons. The ratio of \( M_w \) to \( M_n \) is 4.38 in the dimethyl sulfate-methylated preparation, 2.61 in the derivative prepared.

FIG. 3. Gel Permeation Chromatography of Methylated Kaki-tannins.

A: kaki-tannin, methylated by Kuhn method, \( M_w = 2.48 \times 10^4 \), and \( M_n = 0.95 \times 10^4 \) (the ratio = 2.61).

B: it, done by dimethyl sulfate in 50% aqueous KOH solution, \( M_w = 1.84 \times 10^4 \), and \( M_n = 0.64 \times 10^4 \) (the ratio = 4.38).

C: it, done modified Hakomori method, \( M_w = 1.38 \times 10^4 \), and \( M_n = 0.64 \times 10^4 \) (the ratio = 2.16).
by the modified-Hakomori method. Judging from the highest yield and a narrow molecular weight distribution ($M_w/M_n=2.16$), the methylated preparation by the modified-Hakomori method seems to represent more reasonably the molecular weight of intact kaki-tannin and its distribution than others. The molecular weight of intact kaki-tannin was estimated to be $ca. 1.12 \times 10^4$ in $M_w$ and $ca. 0.52 \times 10^4$ in $M_n$, which was calculated by subtracting the value of the methyl groups in the methylated preparation.

From the above described findings, it may be concluded that kaki-tannin consists of catechin, catechin-3-gallate, gallocatechin and galloccatechin-3-gallate with the ratio of 1, 1, 2, 2, and an unknown terminal residue, and belongs to the proanthocyanidin B groups with a carbon-carbon interflavan linkage between the C-4 of one unit and the C-6 or the C-8 of another unit. It also has the molecular weight of $ca. 1.12 \times 10^4$ daltons in $M_w$ and $ca. 0.52 \times 10^4$ daltons in $M_n$ and appears to have a partial structure of heteropolymer, possibly these four components forming a repeating unit with the ratio of 1, 1, 2, 2, in the molecule.

**EXPERIMENTAL**

Materials and instruments. Immature and green persimmon fruit (Diospyros kaki L., cv. "Hiratane-nashi," astringent type) was harvested in an orchard of Kagoshima University in August, 1975 and 1976. Authentic polyphenols were a gift from Dr. M. Nakagawa, the National Institute of Tea.

Low resolution mass spectra were recorded on a Hitachi RMU-6M mass spectrometer by using a direct inlet system at evaporation temperature between 150 to 180°C and at an ionizing voltage of 70 eV. IR spectra were obtained with a Hitachi IR EPG-G2 spectrometer in KBr and $1H$-NMR spectra at 100 MHz by using (CD$_3$)$_2$CO with or without D$_2$O as a solvent and tetramethylsilane as an internal standard.

Thin-layer chromatography (TLC). Thin-layer chromatograms were developed by the ascending technique at room temperature by using either micro-crystalline cellulose powder (Merk Co.) or silica gel G-60 (thickness: 0.25 mm). In some cases, precoated silica gel plates (Merk Co.) were used for analysis of polyphenols.

Hydrolysis of kaki-tannin with mineral acid. The prepared kaki-tannin was hydrolyzed by heating with 1.5~2.0 N of hydrochloric acid or sulfuric acid for 20 or 60 min in a boiling water bath. After cooling, the solution was extracted with a small quantity of n-butanol and the pigments in the organic layer were analyzed by paper chromatography and cellulose-TLC with Forestal's solvent, (C). Cyanidin and delphinidin were identified by comparing their $R_f$ values and measuring $\lambda_{max}$ in 0.01% HCl-methanol (cyanidin: 535 nm, delphinidin: 546 nm) and the shift of $\lambda_{max}$ after the addition of AlCl$_3$. The quantitative ratio of the both pigments as well as the degraded products with tolene-$\alpha$-thiol were determined from the area of the spots as described below.

Analytical degradation of kaki-tannin with tolene-$\alpha$-thiol and isolation of the degraded products. Kaki-
tannin (50 mg) in a mixture of ethanol (20 ml), toluene-
α-thiol (7.5 ml) and acetic acid (4 ml) was refluxed
under nitrogen. Samples were withdrawn after 0.5, 1, 2, 4, 24,
and 55 hr and analyzed by cellulose- or silica gel-
TLC for the degraded products. The area of each spot
was determined by means of a Shimazu DB
scanner (λ0 = 700 nm, λa = 530 or 520 nm) using a zigzag
scanning technique after coloration with HCl-vanillin
reagent which only reacts with the A ring of the flavan.

After 55 hr-incubation the reaction mixture was
evaporated in vacuo at 35°C. The oily residue was
dissolved into a small amount of methanol. After a
rough removal of toluene-α-thiol by using a Sephadex
LH-20 column with methanol, each product was iso-
lated by silica gel partition chromatography (stationary
phase: 30-50% of ethyl acetate-chloroform mixture
phase: 0.5 M aqueous formic acid solution, mobile
stepwise elution).

Enzymatic hydrolysis of F-2 and F-4. F-2 and F-4
(ca. 10 mg each) in 0.05 M citrate buffer (pH 5.5, 5 ml)
were treated separately with tannase (Sankyo Co.,
from Aspergillus oryzae, 5000 unit/mg of protein) solution
(1 ml) for 60 min at 30°C. After the incubation
the mixture was acidified by 2N H2SO4 and extracted
with stirring (the modified-Hakomori method).11)
Enzymatic hydrolysis of F-2 and F-4
(iii) Kaki-tannin (0.1 g) was dissolved
into dimethyl sulfoxide (10 ml), and powdered sodium
hydroxide (0.1 g) was added in small portions. The
mixture was stirred gently for 2 hr. After another
addition of 2 ml of freshly distilled methyl iodide,
the incubation was continued for 2 hr at room temperature
with stirring (the modified-Hakomori method).11)

Acknowledgements. The authors wish to thank
Prof. N. Takahashi of Tokyo University for valuable
suggestions and the use of his laboratory and equip-
ments in conducting an important portion of this work,
Dr. H. Okazaki of Sankyo Co. (Tokyo) for generous
supply of tannase, Dr. N. Nakagawa of Research
Institution of Tea for providing standard polyphenols,
and The Institute of Japan Spectro. Co. (Tokyo) for
HPLC analysis.

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