Partial Purification, Estimated Chemical Structure, and the Adhesive Property of Castahesion between the Pellicle and Kernel of Chestnut

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Summary

An adhesive substance, H-2, isolated by HPLC is present in the pellicle of the Japanese chestnut which makes it difficult to peel. The adhesive force of H-2 was measured by applying it between two wooden test pieces and its hemagglutination activity using in sheep and rabbit erythrocytes. The hemagglutination of H-2 was not inhibited by sorbitol, glucose, sucrose, glucosamine, N-acetyl-glucosamine or (+)-catechin; its was accelerated by divalent cations, Ca^{2+} , Mg^{2+} , and Mn^{2+} . The pyrolysis-gas chromatogram pattern of H-2 was similar to that of (+)-catechin. The IR spectrum had peaks of 3450 cm^{-1} (OH) and $1605, 1520\text{ cm}^{-1}$ (aromatic ring). The molecular weight of H-2, determined by HPLC, was approximately 1500. These findings indicate that H-2 is a polymer containing (+)-catechin. This adhesive substance between the pellicle and kernel of the chestnut is called "Castahesion".

Key Words: adhesion, castahesion, *Castanea*, chestnut, polyphenol.

Introduction

Studies on the adherence between cells or between tissues in plant have not progressed as far as those in animals, because the natural adhesive substance involved in the adherence has not yet been isolated from plants.

The adhesive mechanism between pellicle and kernel in nuts was studied by comparing the Japanese chestnut, in which the pellicle adheres tightly to the kernel, with the Chinese chestnut, in which the pellicle adheres loosely to the kernel (Tanaka et al., 1981; Tanaka and Kotobuki, 1992a; Tanaka and Kotobuki, 1992b; Tanaka and Kotobuki, 1998). The time required to peel the pellicle from the kernels corresponds to the seasonal change in its phenolic contents (correlation: $r=0.89^{**}$) (Tanaka et al., 1981). The pellicle can be dissolved with chlorous acid which hydrolyzes phenolic substances, allowing the pellicle to be completely stripped off the kernel without damaging the kernel (Tanaka et al., 1981). Histological studies showed that a tannin appeared in the pellicle of Japanese chestnut and migrated to the outer cells and into the space between the pellicle and kernel. During the final growth stage of the nut, the pellicle tightly adhered to the kernel (Tanaka and Kotobuki, 1992b). The investigation with scanning and transmission electron microscopes supported these findings (Hara et al., 1995). Tanaka and Kotobuki (1998) proposed a model of the adhesive process which

involved the deposition of polyphenolic substances containing (+)-catechin between the pellicle and kernel of the chestnut. Thus, the polyphenol in the pellicle was suspected to play an important role in the adhesion. In this report, an adhesive substance is isolated from the pellicle of Japanese chestnut and partially characterized. On the bases of these findings the adhesion of the pellicle to the kernel is discussed.

Materials and Methods

1. Plant Materials

Japanese chestnut (*Castanea crenata* Sieb. et Zucc. cvs. 'Tsukuba' and 'Tanzawa'), and Chinese chestnut (*C. mollissima* Blume cvs. 'Konansho 39' and 'Konansho 50') introduced from the Peoples' Republic of China, were planted in the National Institute of Fruit Tree Science orchard. The two species were crossbred with Japanese and Chinese pollens, respectively.

Pellicles from nuts of the four cultivars were carefully removed using a knife so as not to scar the kernel surface. The peeling time, i.e. the time needed to remove the pellicle from the kernel, was recorded as described (Tanaka et al., 1981).

2. Isolation of Polyphenolic Substances from Pellicle

Pellicles were homogenized in distilled water and the mixture centrifuged at $8000 \times g$. The supernatant was decanted and analyzed for phenolic substances by the Folin-Denis method. The phenolic substances in the water extract were also analyzed by HPLC (model TRI-ROTAR, Japanese Spectrum Co., Tokyo) under the following conditions; column: a reverse phase SC-01

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column ($\phi 4.6 \times 250$ mm) (Japanese Spectrum Co., Tokyo), linear gradient mobile phase: 20 - 80% methanol with 1% phosphoric acid, flow rate: 1 ml/min, detection: UV 280 nm, 0.16 AUFS. The content of phenolic substance in each extract was adjusted to the same level before injection of sample on HPLC. H-1 and H-2 were identified on the HPLC chromatogram of 'Konan-sho 39' and 'Tsukuba', respectively.

3. Test of Adhesive Force

Cubes of the kernel, together with pellicle, were cut from nuts. These were attached, using a commercial adhesive agent, to pieces of wood ($\phi 5$ mm); the adhesive force between the wooden pieces required to remove completely the pellicle from the kernel was measured by a rheometer (Model R-UDJ1015, Ikedarika Co., Tokyo) (Fig. 1).

The adhesive substance, identified as H-2 on the HPLC chromatogram of 'Tsukuba', was concentrated about 120 times to 360 mg/ml. A 10-ml aliquot of the sample was applied to two pieces of wood ($\phi 5$ mm) which were then joined and dried. The adhesive force of H-2 was measured as above.

4. Hemagglutination Assay

After 0.01M phosphate buffer, pH 7.0, containing 0.15M sodium chloride (PBS) was added to sheep or rabbit blood, the sample was centrifuged; the resultant pellet was washed by fresh PBS two times. The water extracts of 'Tsukuba' and 'Konan-sho 39', H-1, and H-2 using the Cooke microtiter system (Dynatech Laboratories, INC., Alexandria) were added to PBS containing 2% sheep or rabbit erythrocyte, and after 30 min at room temperature, the hemagglutination activity was measured (Osawa et al., 1976). The value of hemagglutination activity of samples was expressed as the minimum quantity of agglutination.

Fig. 1. Schematic of an apparatus (rheometer) used for measuring the adhesive force of H-2 in 'Tsukuba' chestnut extracts binding two pieces of wood.

PBS containing a 15 mg/ml solution of sorbitol, glucose, sucrose, N-acetyl-glucosamine, or (+)-catechin was used for the inhibition test of hemagglutination. Twenty five ml of PBS solution containing one of the test chemicals and 25 ml of PBS containing 7 ml (42 mg phenolic substance) of the H-2 were mixed. After standing for 2 hrs at room temperature, 50 ml of PBS containing erythrocyte were added to the solution and the mixture allowed to stand for 30 min longer at room temperature at the end of which time the inhibitory activity of H-2 was measured.

Calcium chloride (CaCl_2), magnesium chloride (MgCl_2), and manganese chloride (MnCl_2) were used for the acceleration test of hemagglutination. Twenty five ml of PBS containing 3.5 ml of H-2, 50 ml of PBS containing erythrocytes, and 0.15M of cation were mixed and allowed to stand for 30 min at room temperature; then the acceleration activity of H-2 was measured.

5. IR Spectrum of H-2

After H-2, purified by HPLC, was lyophilized, the IR spectrum of H-2 on a potassium bromide (KBr) disk was measured by an IR spectrometer (Model 345, Hitachi Co., Tokyo).

6. Pyrolysis-Gas Chromatography of H-2

H-2 and (+)-catechin were analyzed with a Curie point pyrolyzer (Model JHP-2, Japan Anal. Inc., Tokyo) and a gas chromatograph (GC) (Model 5710A, Hewlett Packard Co., Washington D.C.). Pyrolysis was carded out at 670 °C for 3 sec. The GC conditions were as follows: column: 5% FFAP (free fatty acid phase) column ($\phi 4$ mm $\times$ 3 m), column temperature was raised from 80 to 250 °C at 4 °C/min continuously; detection: FID.

7. Molecular Weight Distribution of H-2

The methyl derivative of H-2 was prepared by the method of Hakomori (1964). Distribution of the molecular weights of the methylated H-2 was measured by HPLC on a Shodex GPC A-80M column ($\phi 4.6 \times 500$ mm) (Showadenko Ltd., Tokyo). HPLC conditions were as follows: mobile phase: THF, flow rate: 1 ml/min, detection: UV 280 nm, 0.16 AUFS. Standard acetylated (+)-catechin was synthesized with acetic anhydride/pyridine. Polystyrenes (Chemco Scientific Co., Osaka) were used as molecular standards.

Results and Discussion

The adhesive substance accumulated in the pellicle of the Japanese chestnut before it became tightly bound (Tanaka et al., 1981; Tanaka and Kotobuki, 1992b; Tanaka and Kotobuki, 1998). Therefore, immature nuts were used to purify the adhesive substance. Peeling times and the phenolic contents in the water extracts of the two cultivars of Japanese chestnut and the two cultivars of Chinese chestnut were determined (Table 1).

Table 1. Peeling time and the phenolic content in water extracts of chestnut pellicles.

Cultivar	Harvest date	Pellicle weight	Peeling time	Water soluble phenolics in pellicles
		(g/nut)	(min.sec.)	(mg/nut)
<i>Japanese chestnut</i>				
Tanzawa	Aug. 21	0.95	3' 15" $\pm$ 15.2"	25.3 $\pm$ 0.19
Tsukuba	Sep. 19	1.38	10' 27" $\pm$ 37.7"	96.0 $\pm$ 0.65
<i>Chinese chestnut</i>				
Konansho 50	Oct. 8	0.53	28" $\pm$ 0.6"	7.2 $\pm$ 0.05
Konansho 39	Oct. 11	0.46	25" $\pm$ 0.2"	5.2 $\pm$ 0.02

The average peeling time of Japanese chestnuts was about 15 times longer than that of Chinese chestnuts; the average phenolic contents were 10-fold greater. There is a correlation ($r=0.999^{**}$) between the time for pellicle removal and the phenolic contents in the water extract from the pellicles of the four cultivars. We postulate that the adhesive substance was soluble in the water extract of the pellicle.

The phenolic substances in the water extracts from 'Tsukuba', 'Tanzawa', 'Konansho 39', and 'Konansho 50', analyzed by HPLC (Fig. 2). Revealed peaks at 12 min and 21 min on retention time were obtained in the water extract from Japanese chestnut pellicles. The retention time of the first peak was the same as that of commercial (+)-catechin (Sigma Chemical Co., St. Louis). The second peak in the Japanese chestnut was much higher and comprised of the main water-soluble phenolic substances. The longer peeling time corresponded to the large quantity of phenolics in the second peak, whereas the Chinese chestnut had a shorter peeling time and a correspondingly lower peak. The second peak on the HPLC of 'Konansho 39' and 'Tsukuba' was identified as H-1 and H-2, respectively.

When concentrated H-2 (362.5 mg/ml) was applied to the joining surfaces of two pieces of wood, the adhesive force was 1.2 kg/cm² or 24% of the natural adhesive force between the pellicle and kernel (5.0 kg/cm², Table 2). This method, which seems effective for evaluating adhesive force, indicated that H-2 is the adhesive between the pellicle and the kernel. However, we could not evaluate a weak adhesive force of H-1 extracted from the pellicle of the Chinese chestnut.

Therefore, we applied the hemagglutination assay which determines the agglutination activity of lectin to measure the adhesive activity of phenolic substances. Although no one has studied the agglutination activity between phenolic substances and erythrocytes, we found that the water extract from the pellicle showed hemagglutination activity (Fig. 3). Phenolic substances might be expected to conjugate with carbohydrate and protein existed on cell surface of erythrocytes. Gstimer et al. (1956) and Bate-Smith (1973) reported measuring the content and astringency of tannins extracted from

Fig. 2. HPLC tracings of UV absorption at 280 nm of water extract from chestnut pellicles.
A: Tanzawa B: Tsukuba, C: Konansho 50, D: Konansho 39

Table 2. Comparison of adhesive forces between H-2 and naturally occurring substances between the pellicle and kernel of 'Tsukuba' chestnuts.

Material & cultivar	Adhesive force (kg/cm ²)
H-2	1.2 ± 0.2
Tsukuba	4.9 ± 0.5

Table 4. Inhibition test of hemagglutination assay of H-2

Material	Inhibition
Sorbitol	—
Glucose	—
Sucrose	—
Glucosamine	—
N-Acetyl-d-glucosamine	—
(+)-Catechin	—

Table 5. Acceleration of agglutination by metal ions.

Metal ion	Acceleration
CaCl ₂	+
MnCl ₂	+
MgCl ₂	+

Fig. 3. Photomicrograph showing hemagglutination of erythrocytes in the control (A) and water extract (B) from pellicle.**Table 3.** The phenolic content and hemagglutination assay of water extract from pellicle.

Cultivar	Content of phenolic substance (mg/gFW)		Hemagglutination assay (mg phenolic substance/ml)	
	Water extract	HPLC extract	Water extract	HPLC extract
<i>Japanese chestnut</i>				
Tsukuba	69.6	37.0 ^z	145	6 ^z
<i>Chinese chestnut</i>				
Konansho 39	11.4	4.0 ^y	900	23 ^y

^zH-2 and ^yH-1 were identified on HPLC of 'Tsukuba' and 'Konansho 39', respectively. **Fig. 4.** IR spectrum of a KBr disk of H-2.

plants using extract from erythrocytes.

The hemagglutination activities of water extract and of H-2 at 6 mg phenolic substance/ml from 'Tsukuba' were higher than those of water extract and H-1 at 23 mg phenolic substance/ml from 'Konansho 39' (Table 3). H-2 had the strongest adhesive activity, whereas the water extract from 'Konansho 39' had the weakest. These results indicate that the hemagglutination assay closely approximates the adhesive property.

Sorbitol, glucose, sucrose, glucosamine, N-acetyl-glucosamine, and (+)-catechin did not inhibit the hemagglutination of H-2 (Table 4); whereas the cations of Ca²⁺, Mn²⁺ and Mg²⁺, accelerated the agglutination (Table 5). These results suggest that H-2 is a non-specific adhesive substance whose activity is accelerated by divalent cations.

The IR spectrum of H-2 in a KBr disk gave sharp absorbance peaks at 1605 and 1520 cm⁻¹ to indicate the presence of an aromatic ring and a broad peak at 3450 cm⁻¹ to suggest a hydroxyl group (Fig. 4). An absorbance peak, indicating the presence of a carbonyl group, such as that found in gallic acid, was not detected in the IR spectra of H-2.

These IR spectra were similar to that of procyanidin, 2', 4', 5', 7-tetrahydroxy-flavan-3, 4-diol, from the bark of black spruce (Manson, 1960). When H-2 was hydrolyzed by strong acid, it formed a brown phlobaphene-

Fig. 5. Tracings of pyrolysis-gas chromatogram of H-2 (A) and (+)-catechin (B).

like substance with a procyanidin-color reaction with hydrochloric acid-vanillin solution (Nakabayashi, 1967). Young et al. (1985) reported the acid-induced 'phlobaphene' reaction using (+)-catechin. H-2 was thus conceived to be a catechin-type substance according to the IR spectral analysis and color reaction. Thompson et al. (1972) and Foo and Porter (1980) reported procyanidin polymers composed of only (+)-catechin. Williams et al. (1983) reported the molecular weight distribution of procyanidin polymers in 32 plant species. The dimer, trimer, and oligomer in procyanidin group were extracted from various plants (Hsu et al., 1985; Sun et al., 1987; Hemingway, 1989; Foo and Karchesy, 1989; McGrath and Smith, 1990; Foo and Karchesy, 1991; Ricardo de Silva et al., 1991; Bae, 1994; Prieur et al., 1994).

The composition of fragments obtained by pyrolysis-gas chromatography provided information on the molecular structure of large molecular weight compounds (Tuge, 1978). This method, used to analyze the microstructure of H-2, revealed that H-2 resembled (+)-catechin, which was one of two main phenolic substances in water extract from pellicles of chestnut (Tanaka and Kotobuki, 1998) (Fig. 5). Erman and Lyness (1965) also detected a polymer of substituted catechin-type units from slash pine bark by pyrolysis analysis.

Methylation of H-2 yielded derivatives with molecular weights ranging from several hundred to over a million. The largest peak gave a molecular weight of 1,500 on gel permeation chromatography (Fig. 6).

We suspect that the main element of the polyphenolic substances from pellicles of Japanese chestnut is (+)-catechin and the polyphenolic substances are composed of oligomer forms of (+)-catechin. The affinity of pro-

Fig. 6. Elution pattern of MW distribution for methylated H-2.

cyanidins for proteins is related to the degree of polymerization of the polymers. The large procyanidin oligomers have higher affinities for proteins and form insoluble complexes (Haslam, 1974; Ezaki-Furuichi et al., 1987). However, the (+)-catechin monomer did not conjugate with protein. McManus et al. (1985), Shen et al. (1986) and Spencer et al. (1988) also reported that procyanidins are polar and readily form insoluble com-

plexes with proteins and carbohydrates by hydrogen bonding.

We propose that the adhesive substance, H-2, be designated "Castahesion", for *Castanea* and adhesion. Castahesion is a natural adhesive substance constructed with (+)-catechin in plants. We propose that Castahesion conjugates with proteins or carbohydrates on the cell surfaces of the pellicle and kernel bounding the two.

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ニホングリの渋皮と果肉との接着物質‘カスタヘジョン’の部分精製、推定構造、性質について

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摘 要

高速液体クロマトグラフを用いてニホングリの渋皮に存在する接着物質 (H-2) の部分精製を行った。この物質は、木と木を接着させるとともにウサギの赤血球を凝集させた。この赤血球凝集活性は、ソルビトール、グルコース、ショ糖、グルコサミン、N-アセチルグルコサミン、(+)-カテキンでは阻害されなかった。また、カルシウムイオン、マグネシウムイオン、マンガンイオンの2価のイオンは、凝集活性を促進した。H-2のバイオリス-ガスクロマトグラムパターン

は、(+)-カテキンのパターンと類似していた。IR スペクトルから、 3450 cm^{-1} (OH), 1605 , 1520 cm^{-1} (aromatic ring) に吸収バンドが観察された。これらの結果から、渋皮接着物質 H-2 は (+)-カテキンで構成されるポリマーであると考えられた。HPLC を用いてメチル化した H-2 の分子量を測定したところ $1,500$ にピークが存在した。この渋皮と果肉との接着物質を‘カスタヘジョン’と名付けた。