The Role of Phenolic Substances in the Adhesion between the Pellicle and Kernel of Japanese Chestnut under Experimental Conditions

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Summary
The adhesion process between the pellicle and kernel under heat (62 °C) and hypobaric (4 mm Hg) conditions was analyzed in the immature Japanese chestnut, which is easy to peel. The adhesion process under the experimental condition was similar to that occurring in nature. The peeling time of the nuts heated for 10 hr was about 5 times longer than that of the nuts subjected to hypobaric treatments. Monophenolic substances in the water extracts from pellicle, composed mainly of (+)-catechin and gallic acid identified by GC and GC-MS, diffused to the outer cells of the kernel and the interface between the pellicle and kernel during the first 2 hr of both treatments. There the low molecular weight phenolic substances, including (+)-catechin, polymerized as noted by the shift of their peaks after 2-5 hr of heating. With continued heating, the content of phenolic substances in the alcohol insoluble solids (AIS) fraction of the pellicle increased rapidly and the pellicle tightly adhered to the kernel. Therefore, we postulate that the polyphenolic substances are the adhesive between pellicle and kernel. However, under hypobaric conditions, cell breakdown and migration of the phenolic substances in the tissues occurred during the initial 0-2 hr and ceased. Thus, little or no polyphenolic substances accumulated between the pellicle and kernel to cause their adhesion after 10 hr of treatment.

Key words: adhesion, Castanea, (+)-catechin, chestnut, polyphenol.

Introduction
Although the adhesion between cells and between adjacent organs in plants is an important subject in animals (Alberts et al., 1989), this phenomenon has not been well-documented.

The pellicle of the Japanese chestnut (Castanea crenata Sieb. et Zucc.) harvested at maturity adheres tightly to the kernel, although the ease of pellicle removal of the nuts is influenced by the genetic makeup of the pollen parents (Tanaka and Kotobuki, 1992a). The adhesive force between the pellicle and kernel during early growth and development is weak as in the Chinese chestnut (C. mollissima Blume) which peels easy (Tanaka et al., 1981). Histochemical studies revealed that tannins accumulate in the pellicle cells during the middle of the growth period. The number of tannin cells proliferate in the pellicle as growth continues (Tanaka and Kotobuki, 1992b; Hara et al., 1995). Maximum adhesive force between the pellicle and kernel occurs in the Japanese chestnut when the tannin cells disintegrate and the phenolic substances diffuse and penetrate into the interface between the pellicle and kernel.

The mono- and polyphenolic content in the pellicle of the Japanese chestnut increases rapidly during nut growth, unlike the Chinese chestnut; there is a positive correlation between the pellicle adhesion and the level of phenolic substances in the pellicle (Tanaka et al., 1981). Previously we observed that after the nuts detached from the burrs and fell, the moisture content in the pellicle decreases rapidly and the degree of adhesion between the pellicle and kernel increases rapidly (Tanaka and Kotobuki, 1992b). In the same paper we reported that the moisture content does not influence the adhesion directly, whereas oxygen does.

The adhesion process between the pellicle and kernel in immature nuts (to eliminate effects of growth) exposed to mild heat and a hypobaric stresses to simulate conditions prevailing during later stages of development in nature was studied. The level and roles of phenolic substances in relation to the adhesion process were clarified under these experimental conditions.

Materials and Methods

Peeling Time and Moisture Content of Pellicle subjected to Heat and Hypobaric Treatments

Immature nuts of the Japanese chestnut (cv. Tanazawa), harvested on August 21 in our orchard required only 1 min and 17 sec on an average to remove the pellicle. After removal of the shell, intact kernel with pellicles attached were placed in an incubator at 62 °C (heat treatment) or under a vacuum at 4 mm Hg (hypobaric treatment) using a vacuum drying oven (Teio Scien...
entific Instruments, Co., Tokyo). After given intervals, a subsample of 10 nuts was immediately peeled by the method outlined previously (Tanaka et al., 1981). The peeling time is defined as the time required to separate the pellicle from the kernel with a knife without causing damage. The moisture content of the pellicle was determined by weighing it before and after 2 hr treatment at 130 °C.

Determination of Contents of Total Phenolic Substances and Flavonoid

The pellicles removed from the kernels were immediately placed in a centrifuge tube of distilled water and centrifuged at 7000 g for 10 min. The supernatant was set aside as water extract and the pellet homogenized with 80% methanol (MeOH) and the homogenate centrifuged at 7000 g for 10 min and washed twice with 80% MeOH. The pellet, dried under vacuum, consisted of the alcohol insoluble solids (AIS). The total phenolic contents in the water extract and the methanol extracts were determined using the Folin-Denis method with (+)-catechin as a standard (Nakabayashi, 1967), whereas the flavonoid content estimated using the vanillin-H$_2$SO$_4$ method with (+)-catechin as a standard (Nakabayashi, 1967).

Identification and Quantitative Analysis of (+)-Catechin and Gallic Acid

The monophenolic substances which were recovered from water extracts by partitioning with ethyl acetate after adjustment at pH 4 by IN HCl, were reacted with trimethyl sulfonate (TRI-SIL, Pierce, Rockford) at room temperature. The amount of trimethylsilylated derivatives of (+)-catechin and gallic acid was measured by gas chromatography (GC) and gas-mass spectrometry (GC-MS) with Shimadzu models GC-4B and LKB 9000, respectively (Simadzu Scientific Instruments & Equipment Inc., Kyoto).

The GC conditions were: column, SE-30 column (4 mm ×2 m); column temperature, 200-270 °C with an increase of 2 °C/min; detection, FID; carrier gas, 1 ml/min of N$_2$. The GC-MS conditions: column, SE-30 column (4 mm ×2 m); column temperature, 150-230 °C with an increase of 5 °C/min; carrier gas, 1 ml/min of He. n-Docosan was used as the internal standard.

Thin Layer Chromatography (TLC) of the Water Extract

The water soluble extractives in the pellicle were separated by TLC on a Merck TLC plate silica gel 60 (20×20 cm) using n-butanol : acetic acid : water (2 : 2 : 1 v/v) as a developing solvent. After development, the TLC plate was scanned with a chromatoscanner (Model CS-910, Shimadzu Scientific Instruments & Equipment Inc., Kyoto) at 280 nm.

Molecular Weight Profiles of Phenolic Substances in the Water Extract using Toyopearl HW60F

Aliquots of water extracts of the pellicle were frozen in liquid nitrogen and freeze dried. The residue was dissolved in 0.5 ml of 60% acetone and the solution applied to a Toyopearl HW60F column (2 × 30 cm); the column was eluted with 60% acetone. The fractions containing phenolic substances were collected and their concentrations determined by Folin-Denis method and expressed as (+)-catechin. Sodium polystyrenes molecular weights of 690,000 and 390,000 (Chemco Scientific Co. Ltd., Osaka) and (+)-catechin (Sigma Chemical Co., St Louis) were used as molecular weight standards.

The Content of Phenolic Substances in AIS and AIS from Pellicle

Three hundred mg of pellicle AIS were placed in a test tube containing 5.0 ml of distilled water, 0.5 g of sodium chloride (NaClO$_2$) and 0.3 ml of acetic acid. After the mixture was allowed to react at room temperature for 2.5 hr, 0.5 g of NaClO$_2$ and 0.3 ml of acetic acid were added. After another 2.5 hr, the reaction was stopped and neutralized by 5N NaOH. The solid residue was washed 3 times with distilled water, once with acetone, dried under vacuum, and weighed. Since pheno- nic substances are decomposed oxidatively by sodium chloride and solubilized in water (Ishikawa et al., 1969; Lindgren, 1971) so that the amount of phenolic substances in the AIS was calculated as the difference between the weight of AIS before and after the sodium chloride treatment.

Results

Changes in Peeling Time, Moisture and AIS Percentages of Pellicle

The peeling time of the untreated immature nut of the Japanese chestnut was 1 min and 17 sec (Fig. 1). The peeling time did not increase appreciably during the first 2 hours of heat treatment but it lengthened slightly 2 to 5 hr, but rapidly after 5 hr. The peeling time after 10 hr of heat treatment was 5 min and 42 sec, about 5 times longer than that of the untreated nut. However, peeling time of the nuts after the hypobaric treatment was 1 min and 34 sec, nearly the same as that of the untreated sample.

The moisture content of the pellicle was 65% for the untreated nuts (Fig. 2) and 55% for the heat and hypobaric treated nuts after 2 hr. After 10 hr of heat and hypobaric treatment, the moisture content decreased to 40.8% and 37.5%, respectively.

The AIS content of the untreated pellicle was 13.9% (Fig. 2). During the heat treatment, it increased gradually up to 5 hr and rapidly thereafter until it was 38.9% or 2.8 times that of the untreated nut after 10
hr. The AIS percentage of the pellicle after 10 hr of hypobaric treatment was 21.0%.

Changes in the Total Phenolic and Flavonoid Contents in the Water Extract from the Pellicle

The total phenolic and flavonoid contents in the water extract from the pellicle of the untreated nut were 13.6 and 6.4 mg/pellicle, respectively (Fig. 3). During the initial 2 hr heat treatment, their values increased about 1.5 times. With continuous heating, their contents decreased slowly, then rapidly to 5.9 and 3.0 mg/pellicle after 10 hr, respectively. During the hypobaric treatment, phenolic and flavonoid contents in the pellicle increased rapidly during the first 0.5 hour and then remained constant thereafter.

Changes in the (±)-Catechin and Gallic Acid Contents in the Water Extract from the Pellicle

The monophenolic substances in the water extracts from the pellicle of both treated and untreated nuts consisted mostly of gallic acid and (±)-catechin based on GC and GC-MS analysis (Fig. 4). The (±)-catechin content in the water extract from the pellicle of untreated nuts was 0.56 mg/pellicle, but it reached a maximum of 0.96 mg/pellicle during the first 2 hr of heat treatment (Fig. 5). Thereafter, it decreased rapidly to a minimum of 0.08 mg/pellicle or 14% of the initial value after 10 hr of heat treatment. The (±)-catechin content of the hypobaric treated nuts remained unchanged. There was a negative correlation between the (±)-catechin content in the water extract and the peeling time \( r = -0.81^{**} \). Gallic acid content in the water extract which was 0.26 mg/pellicle in untreated nuts was nearly halved in the 10 hr heat treatment (Fig. 6). Gallic acid level in the hypobaric treated nuts followed a similar trend to that of the heat treatment.

TLC Profiles of the Water Extract from the Pellicle

The amount of bands corresponding to the (±)-catechin increased during the first 2 hr of the heat treatment and then decreased thereafter (Fig. 7). Peaks at low Rfs (0 to 0.35), which might be polyphenolic substances, increased during the first 5 hr of the heat treat-
Fig. 3. Changes in total phenolic substances and flavonoid contents in the water extracts from the pellicle after heat and hypobaric treatments.

The content of phenolic substances of each extract (Total phenolic substances of heat treatment, ○; Total phenolic substances of hypobaric treatment, □) was determined using the Folin-Denis method. The flavonoid content of each extract (Flavonoids of heat treatment, ▲; Flavonoids of hypobaric treatment, △) was determined using the vanillin-H₂SO₄ method.

ment and then decreased thereafter.

Molecular Weight Profiles of Phenolic Substances in the Water Extract from the Pellicle

There were two major peaks in the tracings of the untreated nut, one corresponding to compounds with high molecular weights, between 400,000 and 500,000, the other to compounds with low molecular weights in the range of several thousands (Fig. 8). The total phenolic content estimated from the profile of nuts heat-treated for 0.5 hr was higher than that of the untreated. After a 4-hr exposure, a new peak corresponding to high molecular weight compounds appeared. A similar distribution pattern was found in the 7-hr heat treatment but with a decreased level of phenolics; they continued to decrease through the 10-hr treatment. On the contrary, under the hypobaric condition, no shift of the peak from a lower to a higher molecular weight occurred, although phenolic content in pellicle in the 4-hr treatment nuts was higher than that of the untreated but equal to that of the 4-hr heat-treated pellicles.

Changes in AIS Weight and the Weight of Phenolic Substances in the AIS from the Pellicle

The AIS weight and the weight of the residual phenolic substances in the AIS in pellicles from the untreated nut were 102 and 62 mg/pellicle, respectively (Fig. 9). The former was 60% of that from the unextracted pellicle of the untreated nut. The values increased rapidly after 5 hr of heat treatment and reached 172 and 141 mg/pellicle, respectively after 10 hr or equivalent to 1.7 and 2.3-fold increases, respectively. The gain in the AIS weight during 10 hr heat treatment paralleled that of the gain in phenolic substances. However, the AIS and phenolic substances per pellicle under hypobaric conditions decreased slightly for 2 hr and then remained constant. There was a positive correlation between the weight of phenolic substances in the AIS from the pellicle and the peeling time (r=0.89**).

Discussion

The phenolic content in the pellicle in the Japanese
Fig. 5. Changes in (+)-catechin content in the water extracts from the pellicle after heat and hypobaric treatments. The content of (+)-catechin of each extract (Heat treatment, ●; Hypobaric treatment, ○) was measured by GC and GC-MS.

Fig. 6. Changes in gallic acid content in the water extracts from the pellicle after heat and hypobaric treatments. The content of gallic acid of each extract (Heat treatment, ●; Hypobaric treatment, ○) was measured by GC and GC-MS.

Fig. 7. TLC tracings of the water extract from the pellicle after heat treatments. Absorbance recorded at 280 nm.

Chestnut increased rapidly during nut growth unlike in the Chinese chestnut (Tanaka et al., 1981). This suggested that the phenolic substances played an important role in the adhesion between pellicle and kernel of the nuts, but their relationship, e.g., the behavior and polymerization of phenolic substances in the adhesion process, was not clear. With freshly harvested nuts, the peeling time correlated negatively with moisture content of the pellicle but positively with the AIS content in pellicle (Tanaka and Kotobuki, 1992b). The oxygen also influenced the adhesion process of the Japanese chestnut (Tanaka and Kotobuki, 1992b).

The pellicles of immature nuts contain phenolic substances during rapid growth are easy to remove. But with heat-treated nuts in ambient atmosphere or those placed under hypobaric condition, the peeling time correlated positively with the pellicle AIS (r=0.95 **) but negatively with the moisture content (r=-0.62 **). These relationships correspond to those obtained under natural conditions (Tanaka and Kotobuki, 1992b).
Phase I: Cell Breakdown Period

Although the peeling time did not increase during the initial 2-hr heat treatment, the moisture content of the pellicle decreased. Simultaneously, the total phenolics, including (+)-catechin and gallic acid, and flavonoid contents in the water extracts increased. These substances are probably exuded from the pellicle surface. These results indicate that during phase I, the inner layer of the pellicle cells was disrupted by dehydration under both heat and hypobaric conditions allowing phenolic substances in the vacuoles to diffuse to the interface between the pellicle and kernel.

Phase II: Polymerization Period

The phenolic contents in the molecular weight profiles reached a maximum value after heat- and hypobaric treatments for 4 hr. However, in the heat treatment, the distribution of phenolic substances shifted from low molecular to high molecular weights in phase II; this shift did not occur under hypobaric condition. These findings suggest that the phenolic substances in the pellicle polymerized during the heat treatment, whereas they did not under the hypobaric condition.

The level of (+)-catechin, a main component of the phenolics in heat treated pellicle, decreased after phase I, whereas its content under the hypobaric treatment and that of gallic acid under both treatments did not decrease after phase I. Hence, we assumed that (+)-catechin is the main structure of total polyphenolic substances.

Phase III: Adhesion Period

The adhesion between the pellicle and the kernel became tighter during phase III. The concentrations of (+)-catechin, total phenolic substances and flavonoid in the water extracts decreased considerably after 10 hrs of heat treatment. On the other hand, the AIS weight and the weight of the phenolic substances in the AIS of the pellicle increased significantly. A high positive correlation between the AIS weight and the peeling time exists (r=0.89**), which indicates that the polyphenolic substances, which polymerized during phase II, are responsible for the adhesion.

The Model of Adhesion Process of the Japanese Chestnut

Based on the above findings, we propose that the pellicle cells are disintegrated by dehydration during phase I, allowing (+)-catechin, a precursor of the adhesive substances, to diffuse to the interface between the
Fig. 9. Changes in alcohol insoluble solids (AIS) weight and the content of phenolic substances in the AIS from the pellicle after heat and hypobaric treatments. AIS of heat treatment, ○; AIS of hypobaric treatment, □. Phenolic substances of heat treatment, ▲; Phenolic substances of hypobaric treatment, △) was calculated as the difference between the weight of AIS before and after the sodium chloride treatment.

pellicle and kernel and accumulated. In phase II, (+)-catechins polymerized and become adhesive, binding the pellicle to the epidermal cells of the cotyledons. Polyphenolic substances, which consist of hydrogen, ionic and covalent bonds are known to bind with proteins and other natural polymers (Haslam, 1974, 1977; McManus et al., 1985; Shen et al., 1986; Ezaki-Furuchi et al., 1987; Spencer et al., 1988; Macheix et al., 1990). In phase III, the polyphenolic substances thus conjugated at the interface make it difficult to remove the pellicle.

In summary, the phenomenon of the adhesion between pellicle and kernel of the nuts undergo the following biophysical changes: The polyphenolic content in the pellicle of the Japanese chestnut increases rapidly during the nut growth (Tanaka et al., 1981). These phenolic substances are synthesized in the epidermal cells of the pellicle. The enlargement of the kernel causes these tannin cells to disintegrate forcing their contents to spread into the interface as shown by our histochemical study (Tanaka and Kotobuki, 1992b). Oxygen accelerates the adhesion between pellicle and kernel of the nut (Tanaka and Kotobuki, 1992b). Thus, we hypothesize that the phenomenon of the adhesion in nature occurs in three phases: I) cell break down by dehydration, II) polymerization of phenolic substances, and III) adhesion of the pellicle to the kernel.

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Literature Cited


人為的処理によるニホンギの渦皮と果肉の接着過程におけるフェノール物質の役割

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摘 要

ニホンギの渦皮と果肉が強く接着する現象の解明を目的とし、また、接着の弱い未熟のニホンギを用いて加熱処理（62℃）および減圧処理（1 mmHg）を行った。この実験条件は、自然条件下で起こるクリ果実の渦皮と果肉との接着過程を再現するためのモデル実験として設定した。加熱処理を行った果実は、10時間処理後に、渦皮が軟化し、渦皮と果肉の接着が困難となった。加熱処理区では、処理開始から2時間目までに渦皮の細胞にあった（＋）-カテキンと没食子酸等の低分子フェノール物質が、渦皮と果肉の間に移動した。その後、2～5時間の間に低分子のフェノール物質が高分子化した。5～10時間の間には、アルコール不溶性部分（AIS）に含まれるフェノール物質の量が急激に増加し、渦皮と果肉の剝皮が困難となった。このことから、ポリフェノール物質の渦皮と果肉との接着物質であると考えられた。しかしながら、減圧処理区は、2時間目までは加熱処理区と同様にモノフェノール物質が渦皮と果肉の間に移動したが、加熱処理で認められた2時間目以降の変化は認められなかった。そのため、高分子フェノール物質が形成されずに、渦皮の剝皮が10時間処理後も容易であったと考えられた。