A Model Experiment for De-astringency of Persimmon Fruit with High Carbon Dioxide Treatment: *in vitro* Gelation of Kaki-tannin by Reacting with Acetaldehyde

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In order to explain the mechanism for removing the astringency from persimmon fruit by high carbon dioxide treatment, we studied *in vitro* whether purified kaki-tannin, a kind of polymeric proanthocyanidin, reacts with acetaldehyde to become a gel under mild conditions or not.

Kaki-tannin reacted with acetaldehyde in a relatively short time to become a gel in phosphate buffer at pH 6 to 8. Phosphate, malate and citrate accelerated the gel formation, whereas ethanol, ascorbate and Tricine buffer prevented it. Formaldehyde was more effective, and propylaldehyde was less effective than acetaldehyde in causing gelation.

We suggest that the de-astringency is due to the insolubilization of kaki-tannin, which occurs by means of reacting with acetaldehyde produced in the fruit during high carbon dioxide treatment.

Japanese persimmon fruit, such as cv. "Hiratanenashi" and "Yokono," has remarkable astringency even at a mature stage, so it is only edible after removal of the astringency by some treatments or as a dried fruit.¹⁾ We have studied the de-astringency of persimmon fruit with high carbon dioxide and have reported on an improved method which produced non-astringent fruit of excellent quality.²⁾ This method also provided proof of the important role of acetaldehyde which was produced in the flesh during the carbon dioxide treatment.³⁾ This importance has been further supported by the following findings; i) in a normal atmosphere, an application of dinitrophenol or arsenic trioxide to astringent persimmon fruit induced the insolubilization of kaki-tannin⁴⁾ (it caused remarkable astringency of the fruit). The insolubilization was examined by the "tannin print" method using FeCl₃-impregnated filter paper.^{3,5)} It was described by Beevers⁶⁾ in 1961 that dinitrophenol and arsenic trioxide caused a high accumulation of acetaldehyde in plant tissues without other anaerobic treatment. ii) Soluble tannin in fruit heated in a boiling water bath

for an appropriate time was converted to an insoluble form by the application of formaldehyde or acetaldehyde vapor, but not of ethanol vapor.^{4,7)}

On the other hand, kaki-tannin was recently explained to be a kind of polymeric proanthocyanidin.⁸⁾ There have been few reports on the chemical properties of proanthocyanidins. Therefore, to study the mechanism of de-astringency we have investigated the mechanism of the insolubilization and gelation of kaki-tannin during the carbon dioxide treatment.

The experiments presented here investigated whether acetaldehyde reacts with kaki-tannin *in vitro* under a mild physiological condition to form a gel (*i.e.* to cause its insolubilization) and examined several factors influencing the rate of gelation and gel toughness.

MATERIALS AND METHODS

Young persimmon fruits (*Diospyros kaki* Linn., cv. "Hiratanenashi" and "Jiro") were harvested from July to August in 1980, at the Toso Orchard of Kagoshima University, and cv. "Yokono" and "Fuyu" at the Kihoku

Branch of the Wakayama Fruit Tree Experimental Station. They were very young fruit and the average fruit weight was about 20 to 30 g.

Kaki-tannin was prepared from the fruit of each cultivar by the same procedure which has been described in detail in a previous paper.⁹⁾ The aqueous kaki-tannin solution for the gelation experiments was usually adjusted to about 1.5 to 2.0% of the tannin content, being determined by the Folin-Ciocalteu method¹⁰⁾ using (+)-catechin as a standard for the tannin. This concentration of kaki-tannin was convenient for various operations and storing and allowed us to observe the gelation processes at a proper rate.

Acetaldehyde (a special grade for biochemical experiments) was used for the gelation of kaki-tannin and was purchased from Merck Co. Trimethylacetaldehyde was purchased from Aldrich Chem., Co. and (+)-catechin, a standard for the tannin determination was purchased from Sigma, Co. MES and Tricine, for Good's buffer, were purchased from Nakarai Chem., Ltd. All other chemicals were purchased from Wako Pure Chem., Ind. Ltd.

The rate of gelation and the gel point were observed by monitoring the increase in viscosity and in optical density (O.D.) at 610 nm. The measurement of viscosity of the kaki-tannin solution during gelation was carried out using a B-8 H type viscosimeter with a BL rotor (at 2.5 or 10 rpm, Tokyo Keiki Seizoshō, Co., Ltd.).

RESULTS

Increase in viscosity and turbidity during gelation

As a preliminary experiment, the purified kaki-tannin (*ca.* 1.7%) was incubated in a phosphate buffer (pH 6.5) containing acetaldehyde at 25°C. The gelation process of the reaction mixture was observed by measuring the increase in viscosity and turbidity. The results are shown in Fig. 1. Within 20 min after the reaction had started, a slight turbidity could be detected without any increase in viscosity. An abrupt increase in viscosity was observed at 80 min and at the same time the turbidity continued to increase in an arithmetic progression. The solution was transformed into a flexible and tough gel at 120 min after the incubation started.

From these results, it was thought that observation of the increase in O.D. at 610 nm during gelation was more useful than measurement of the increase in viscosity for investigating the gelation process of kaki-tannin. Therefore, the former O.D. observation was

FIG. 1. Changes of Turbidity and Viscosity during Gelation of Kaki-tannin by Reacting with Acetaldehyde.

The reaction mixture, consisting of kaki-tannin (1.47%, w/v), acetaldehyde (0.98%, w/v) and phosphate buffer (30 mM, pH 6.5), was incubated at 25°C. The turbidity of the solution was examined as the increase in O.D. at 610 nm (—○—) and the increase in viscosity (—●—) was measured by a viscosimeter. An asterisk indicates the point when a completed gel was recognized with the naked eye.

FIG. 2. Effect of pH on Gelation of Kaki-tannin by Reacting with Acetaldehyde.

The reaction mixture, consisting of kaki-tannin (1.15%, w/v), acetaldehyde (0.98%, w/v) and phosphate buffer (30 mM, pH ranging from 2.0 to 8.0), was incubated at 25°C. A tough gel resulted within 1 hr at pH 2.0. An asterisk indicates the point when a completed gel was recognized with the naked eye.

applied to analyse the gelation phenomenon.

Effect of pH on gelation

The rate of gelation of kaki-tannin by reacting with acetaldehyde was definitely influenced by the pH of the incubation medium, in this case, the phosphate buffer. Figure 2 shows the increase of turbidity in 1 hr, when the kaki-tannin (58.5 mg) was incubated at 25°C in 4 ml of phosphate buffer (finally 30 mM), pH rang-

ing from 2.0 to 8.0, containing 39.4 mg of acetaldehyde. At and below pH 3.5, rapid turbidity was observed and tough gel resulted for a short time, especially at pH 2.0 within 1 hr. The gelation reaction, which showed a minimum rate at pH 4 and 5, was stimulated in the range from pH 6 to 8. The rate of gelation at pH 7.0 was nearly the same as it was at pH 3.5. At and over pH 6.0, the resulting gel had a slightly brown color and as time elapsed the brown color became more gradually intense. At the more acidic pH the gel remained creamy or white in color.

Effect of acetaldehyde content on gelation

Figure 3 shows how the content of acetaldehyde influences the rate of gelation under the conditions of a given kaki-tannin content and buffer concentration (1.1%, 30 mM, pH 6.5). The rate of increase in turbidity was very dependent on the acetaldehyde content. The reaction mixture with a ratio of kaki-tannin to acetaldehyde of about 1:1 (w/w) showed a striking increase in turbidity soon after the reaction started and was transformed into a gel after 3 hr incubation at 25°C. At one-half of this amount of acetaldehyde in the reaction mixture it required 5 hr to change the solution

into a gel. Four days after the reaction started, the reaction mixture with a ratio of about 50 to 1, kaki-tannin to acetaldehyde, became fairly turbid but not a gel. At a low content of acetaldehyde the gel formed was relatively soft.

Effect of molar concentration of phosphate buffer on gelation

The effect on gelation of the molar concentration of the phosphate buffer was examined at pH 6.5 in the same reaction mixture as Fig. 3. The results showed that in spite of the addition of a sufficient amount of acetaldehyde, the concentration of the phosphate buffer strongly influenced the rate of gel formation and the increase in turbidity (Fig. 4).

The reaction mixtures containing 300 mM and 120 mM of phosphate buffer became turbid at almost the same rate and formed a gel at 1.5 hr and 2 hr of incubation respectively. The reaction mixture containing the 30 mM phosphate buffer formed a gel after 3 hr and in the 6 mM phosphate buffer, gel formation was recognized after 19 hr. The gel resulting in the reaction mixture containing 6 mM phosphate buffer was transparent. Within 24 hr the reaction mixture without the phosphate buffer showed a slight turbidity.

FIG. 3. Effect of Acetaldehyde Content on Gelation of Kaki-tannin.

Each incubation mixture (4 ml) contained kaki-tannin (42 mg), acetaldehyde (the content ranging from 0.8 mg to 39.4 mg) and phosphate buffer (30 mM, pH 6.5). The incubation was carried out at 25°C in a sealed test tube and cell. The measurement was done as shown in Figs. 1 and 2. ○, 39.9 mg of acetaldehyde; ●, 19.7 mg; △, 7.9 mg; ▲, 3.9 mg; □, 1.9 mg; ■, 0.8 mg.

FIG. 4. Effect of Molar Concentration of Phosphate Buffer on Gelation of Kaki-tannin by Reacting with Acetaldehyde.

The reaction mixture consisted of kaki-tannin (1.15%, w/v), acetaldehyde (0.98%, w/v) and phosphate buffer (the molar concentration ranging 0 to 300 mM, at pH 6.5). See Figs. 1, 2 and 3 for details. □, 300 mM; ▲, 120 mM; △, 30 mM; ●, 6 mM; ○, 0 mM, only H_2O .

FIG. 5. Effect of Buffer Constituents on Gelation of Kaki-tannin by Reacting with Acetaldehyde.

The reaction mixture contained kaki-tannin (1.15%, w/v), acetaldehyde (0.98%, w/v) and 30 mM buffer having different constituents. See Figs. 1, 2 and 3 for details. ■, $\text{Na}_2\text{HPO}_4\text{--H}_3\text{PO}_4$ and $\text{Na}_2\text{HPO}_4\text{--citric acid}$ buffer at pH 7.0; △, citrate at pH 6.0; ▲, phosphate at pH 6.0; □, MES-NaOH at pH 7.0; ●, Tris-AcOH at pH 7.1; ○, Tricine-NaOH at pH 7.4.

Effect of buffer constituents on gelation

Changes in the increase of turbidity caused by different buffer constituents were examined in the next experiment at a given molar concentration (30 mM). The rate of increase in turbidity accompanied by gel formation was considerably different in each buffer system (Fig. 5). At pH 7.0, the buffers $\text{Na}_2\text{HPO}_4\text{--H}_3\text{PO}_4$ and $\text{Na}_2\text{HPO}_4\text{--citric acid}$ had the same rate of increase in turbidity and formed a gel at 2 hr of incubation. The citrate buffer (pH 6.0) stimulated formation of the gel more rapidly than the phosphate buffer with the same pH. The gelation of kaki-tannin was accelerated less in the buffer of MES-NaOH (pH 7.0) or Tris-acetate (pH 7.1) than in the former buffers. No detectable increase of turbidity was observed within 24 hr in the reaction mixture consisting of Tricine-NaOH buffer (pH 7.4).

Reactivities of several aldehydes with kaki-tannin

Some aldehydes having a proton(s) at the α -position, other than acetaldehyde, caused gel formation of kaki-tannin under a mild condition (30 mM phosphate buffer, pH 6.5, at 25°C), as shown in Fig. 6. Formaldehyde caused an extremely rapid gelation within 30 min. Propylaldehyde, having an extra meth-

FIG. 6. Reactivities of Several Aldehydes with Kaki-tannin in the Phosphate Buffer at pH 6.5.

The reaction and measurement were carried out as in Figs. 2 and 3. —*, formaldehyde; —●—, acetaldehyde; —■—, propylaldehyde; —▲—, trimethylacetaldehyde. The incubation mixture contained the same amount of each aldehyde (39.4 mg/4 ml).

FIG. 7. Effect of Several Additives on Gelation of Kaki-tannin by Reacting with Acetaldehyde.

Each concentration of several additives was 100 mM, except 12% of ethanol and 29.6 mM of dimedone. The reaction and measurement were carried out as in Figs. 2 and 3. ■, NaCl; ▲, Na_2 malate; ●, glucose and buffer only; □, CaCl_2 ; ○, EtOH; △, Na ascorbate and dimedone.

ylene group than acetaldehyde, required more time to make a gel, at 45 hr of incubation. Trimethylacetaldehyde, having no proton at the α -position, did not induce gelation of kaki-tannin. Even after 7 days, an increase in turbidity was not detected from the increase in O.D. at 610 nm.

Effect of several additives on gelation

The influence of several additives to the reaction medium on the gelation of kaki-tannin was examined. Glucose (100 mM)

showed no effect on the acceleration of gelation but the same amount of sodium malate strongly stimulated it and the reaction medium transformed into a gel after 3.5 hr (Fig. 7). On the other hand, ethanol (12%), sodium ascorbate (100mM) and dimedone (29.6mM) prevented the reaction. The reaction mixture containing 12% ethanol resulted in a soft gel after 2 days, but the mixture containing sodium ascorbate and dimedone did not result in turbidity and a gel even after 7 days. Salting-out predominated the acceleration of gelation in the action of sodium chloride (100mM). The solution resulted in a white precipitate for a short time and thereafter formed a gel at about 4.5 hr after the reaction started.

Reactivities of three kinds of kaki-tannin with acetaldehyde

It is important to know whether kaki-tannin prepared from the non-astringent type fruit transformed into a gel after adding acetaldehyde in the same manner as that from the astringent type fruit, cv. "Hiratanenashi." Kaki-tannins prepared from cv. "Fuyu," non-astringent type and from cv. "Yokono," astringent type fruits formed turbidity at the same rate and in similar manner, as shown in Fig. 8. "Fuyu"-tannin became a gel more rapidly than the other two kaki-tannins which

were prepared from two kinds of astringent fruits. All of them transformed into a gel within 8 hr, at about the same tannin concentration (Hiratanenashi; 1.15%, Yokono; 0.93%, Fuyu; 1.12%).

DISCUSSION

The present work on de-astringency was carried out *in vitro*, in contrast with all the other works which were done *in vivo*. We examined whether the purified kaki-tannin could react with acetaldehyde to form a gel under a mild condition, a physiological condition. It has been known for a long time that large amounts of acetaldehyde accumulated in the flesh of persimmon fruit when de-astringency was completed by some treatments, e.g. high carbon dioxide,^{11~14)} ethanol^{11,12)} and warm water.^{7,15)}

At pH 6 to 7, a physiological pH presumed generally as the pH of plant cells, kaki-tannin easily became a gel by reacting with acetaldehyde (in the weight ratio of about 1 to 1), as shown in Figs. 1 and 2. The reaction was strongly dependent upon the pH of the medium and the minimum rate was recognized between pH 4 and 5 (Fig. 2). This finding was consistent with the report described by Hills and Urbach¹⁶⁾ in that the reaction of phloroglucinol or the A-ring of catechin with the relatively weak electrophilic aldehydes was slowest at pH 4.5.

In considering the function of acetaldehyde on de-astringency, it is interesting that the other kinds of aldehydes behave very differently in their reaction with kaki-tannin, as shown in Fig. 6. The fast reaction of formaldehyde, the slow reaction of propylaldehyde and the non-reaction of trimethylacetaldehyde suggest that the reaction between kaki-tannin and acetaldehyde contains a "condensation reaction." It is probably a reaction similar to phenol-formaldehyde condensation known popularly in polymer chemistry. We have already explained kaki-tannin to be a kind of polymeric proanthocyanidin.⁸⁾

Singleton and Esau¹⁷⁾ stated that during

FIG. 8. Reactivities of Three Kinds of Kaki-tannin with Acetaldehyde.

The reaction and measurement were carried out as in Figs. 2 and 3. The tannin concentration of each medium is; Fuyu; 1.12%, Yokono; 0.95%, Hiratanenashi; 1.15%. ○, Fuyu-tannin; ▲, Yokono-tannin; ●, Hiratanenashi-tannin.

wine aging, phenols and anthocyanin might react with acetaldehyde to form a haze and precipitate. Some of the polyphenols in wine are found to be low molecular weight flavonoids and procyanidins.¹⁸⁾ Similarly, the addition of formaldehyde to the mash prior to the brewing of beer produced a great reduction in the tendency of the beer to form a haze by precipitating anthocyanogens.^{19,20)}

An important problem remains obscure: whether the insolubilization of kaki-tannin *in vivo* is responsible for the formation of a gel in the tannin cells. There is some evidence in regard to this point.

Gazit and Levy,²¹⁾ Kitagawa,²²⁾ and Yoshimura and Kusumoto²³⁾ illustrated a tannin cell filled with insoluble tannin in fruits from which the astringency was removed by some treatments. We also observed that the isolated tannin cells prepared by using cellulase and Macerozyme were transparent and colorless which looked to be filled with a gel-like substance. Kitagawa²⁴⁾ described that the tannin cells in the non-astringent fruit were broken and cracked like glass when he crushed them beneath a cover glass.

The insoluble tannin in the non-astringent cells was observed to be transparent and colorless, as described above. The present experiment showed that at a high buffer concentration a turbid gel was formed but at a lower concentration clear gel formed. It seems that the ion concentration may be relatively low in the central vacuoles of tannin cells.

The fundamental change in the tannins leading to insolubility and the mechanism initiating this change is suggested by these findings that the kaki-tannin transforms into a gel by reacting with the acetaldehyde produced by the fruit cells under high carbon dioxide. At least, it can be evidently concluded that it has a character which reacts easily with acetaldehyde in the presence of some acids, such as phosphate, malate or citrate (Figs. 4, 5 and 7).

The importance of acetaldehyde has further been supported by the following results.

Kitagawa²⁴⁾ tried to confirm it through a unique experiment. He boiled some immature

fruits to inactivate all the enzymes but some quantity of tannin remained in a soluble form. When the fruits were then treated with ethanol, methanol or acetaldehyde, only the acetaldehyde effectively caused insolubilization of the residual soluble tannin and removed the astringency. We have also ascertained the importance from a similar experiment *in vivo*.⁴⁾

Figure 3 shows that at least more than 2% of acetaldehyde for a given amount of kaki-tannin is essential for gelation. In this experiment the concentration of kaki-tannin was 1.15%, while the persimmon fruit has an average 1.5 to 2.0% tannin.¹⁾ Thus the content of kaki-tannin in the tannin cells is assumed to be around 6.0% on the fresh weight basis. If it is correct, the kaki-tannin in these cells may become a gel more easily at a lower acetaldehyde content. This may be the amount of acetaldehyde which the persimmon fruit can produce itself under such a de-astringency treatment. Nakamura¹²⁾ reported that 8.3 mg% of acetaldehyde accumulated in the flesh after completing de-astringency by a high carbon dioxide treatment (7 days at about 20°C).

The striking fall in astringency during the growth and maturing on non-astringent type fruits of persimmon can be observed and they are edible as fresh fruits without any treatment.¹⁾

Figure 8 shows that "Fuyu"-tannin forms turbidity at the same rate and in similar manner to "Yokono"- and "Hiratanenashi"-tannin. Whether "Fuyu"-tannin has a chemical tendency to become a gel more rapidly is obscure. It is generally known among persimmon producers that the "Yokono" fruit is one which does not easily become completely non-astringent after the common treatments. Figure 8 showed evidence that "Yokono"-tannin reacted with acetaldehyde more easily than "Hiratanenashi"-tannin. The difference between both tannins is not known to be physiologically significant at the present time. It appears to be very important to investigate the derivation of the difference in the reaction

with acetaldehyde. New information is needed from a further *in vivo* examination of this point.

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