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Effect of Ingestion of the Japanese Persimmon 'Saijo' Fruit on Ethanol Levels in the Blood of Humans and Rats

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The Japanese astringent-type persimmon 'Saijo' fruit is considered to contain bioactive compounds that help to alleviate the deconditioning seen after excessive intoxication with alcohol in humans. To evaluate the contribution of ingestion of persimmons in lowering blood ethanol levels in intoxicated humans, we investigated the blood ethanol concentration with the ingestion of persimmons (fresh or semi-dried persimmon fruit and persimmon extract) before or after alcohol consumption (including a comparison with that of apples before alcohol consumption). For rats, we investigated their blood ethanol concentration with the ingestion of persimmon extract before alcohol administration, and also detected the antioxidant activity (measured as ferric reducing antioxidant potential (FRAP)) and flavonoid content in their blood after administration of persimmon extract. Ingestion of persimmons before alcohol consumption significantly reduced human blood ethanol levels compared with control values 1 h after alcohol consumption, and this was even more effective than that observed with apple ingestion. Rat data also suggested the same tendency of persimmon extract, but the difference was not significant. There was no significant difference in the human blood level of ethanol between ingestion of persimmons after alcohol consumption and that of control. In addition, neither an increase in the level of flavonoids nor an increase in antioxidant activity were detected in rat plasma; there was even a slight decrease in FRAP after administration of persimmon extract. Taken together, these data showed that the functional compound kaki-tannin alone or together with other components in persimmon fruit ingested by humans before alcohol consumption was effective in lowering blood ethanol levels. These components are not entirely absorbed to blood capillaries, so they might adsorb the ethanol in the human digestive system to depress the absorption coefficient of ethanol on the surface of the gastrointestinal epithelium.

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Key words: *'Saijo' persimmon, drunken sickness, blood ethanol levels, kaki–tannin, gastrointestinal epithelium*
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Persimmon (*Diospyros kaki* Thunb.) has been cultivated in Japan for several centuries, and is thought to have originated in Southern China[1]. It contains many bioactive compounds, including carotenoids, tannins, sugars, hydrocarbons, lipids, aromatics, flavonoids, terpenoids, steroids, naphthoquinones, amino acids[2] and minerals[3]. It is also a good source of nutritional antioxidant polyphenols[4),5)], β-carotene[6] and dietary fiber[3]. The fruit and leaves of the persimmon are used to treat coughs[2] and hypertension[7),8)]. The fruit and leaves of the persimmon as well as extract from the peel also have several health-promoting effects. These include : free radical-scavenging activity[9] ; prevention of a rise in the level of lipids in plasma[10] ; anti-oxidative effects (from the whole fruit[3]) ; an improvement in lipid levels in plasma and the liver (from compounds in the leaves[11]) ; anti-oxidative and anti-genotoxic effects (from compounds in the peel extracts[12] ; and an anti-mutagenic effect against ultraviolet irradiation (found in kaki-shibu (crude kaki-tannin extract) and its tannin-degraded products[13]). Using gas chromatography (GC), OGATA[14] showed that Japanese persimmon 'Fuyu' (sweet-type) fruit juice (including the peel) significantly decreased the ethanol level in blood and alleviated excessive drunkenness in rabbits after alcohol administration. OGATA suggested that the pectin and/or kaki-tannin contained in persimmon fruit juice may "coat" the gastrointestinal epithelium and exert an uptake-inhibitor effect. KOIKEDA *et al*.[15] reported that taking 'Saijo' persimmon extract before drinking alcohol could significantly help in lowering the ethanol concentration in blood for people who have a low tolerance for alcohol.

In the present study, we examined the activity of 'Saijo' persimmon in lowering the concentration of ethanol in blood after administration of the whole fruit or fruit extract to humans or rats. We also established a time-course for absorption of the persimmon extract from the gastrointestinal epithelium to the blood vessels of rats. Using a ferric-reducing antioxidant power (FRAP) assay, we also examined changes in antioxidant activity in the plasma of rats after administration of persimmon extract.

Materials and Methods

1. Ethical approval of the study protocol

The animal studies were approved by the Ethics Committee for Animal Experimentation at Shimane University (Matsue, Japan). The animals were handled according to institutional guidelines. All subjects provided written informed consent to participate in all experiments in compliance with the certification of the Shimane University Health Administration Center.

2. Reagents and solutions

Ethanol (99.5%) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Distilled water was used to prepare the solutions. The standard curve for calculation of ethanol concentration was prepared in the range of 0.0～400 ppm (326μg/mℓ) using samples of whole blood obtained from each human subject before administration of fruit or alcohol. All standards were prepared at 0℃. Artificial stomach liquid was prepared with 0.2% NaCl (Wako), 0.32% pepsin (Sigma-Aldrich, St Louis, MO, USA) and adjusted to pH 2 by HCl (0.1% final concentration)[16),17)]. Purified kaki-tannin was obtained from Maruzen Pharmaceuticals Company Limited (Onomichi, Japan).

3. Plant materials

(1) Fresh persimmon fruit We used the mature persimmon (*Diospyros kaki* Thunb. 'Saijo') fruit after removal of astringency. The fruits used were of uniform weight (～200 g/fruit, containing～1.5% kaki-tannin), without visual defects, and were harvested in Shimane prefecture (Japan). For the measurement of polyphenol content, 'Saijo' persimmon harvested from farm fields in the Shimane prefecture were used. Astringency was removed from fruit by dry ice (1.5% of total fruit weight).

(2) Semi-dried persimmon fruit The fresh persimmon 'Saijo' fruit is available only from October to December. It is not available for experimentation during other seasons. JUNG *et al*.[6] found that the contents of dietary fibers and trace elements in fresh and equivalent quantities of dried persimmon fruit were comparable, and proposed that the latter could substitute for the fresh fruit. We therefore used the semi-dried persimmon fruit (containing 32% of the water content of the fresh fruit) made from the fresh, mature persimmon. This semi-dried fruit was used in the experiments carried out in winter in place of the fresh persimmon 'Saijo' fruit.

(3) Persimmon extract The fruit extract was used in the experiments carried out in winter as a substitute for the persimmon fruit. Fruit without

calyces were washed and crushed in a food processor or blender. The crushed fruit was extracted in hot water for 30 min and filtered through a clean cloth. The filtered extract was subsequently centrifuged at 3,000 rpm for 10 min at room temperature, and the supernatant (designated 'persimmon extract') stored at −20℃ until use. A 35-mℓ persimmon extract was equivalent to ~ 200 g of fresh fruit based on the amount of tannin content.

4. Animals

Male WKY / Izm rats (SLC, Incorporated, Hamamatsu, Japan) were used for the alcohol administration experiment. Male Wistar rats (Charles River Laboratories, Tokyo, Japan) were used for the FRAP assay and extraction of flavonoids from plasma. All animals were housed at the Institute of Animal Experiment of Shimane University with a controlled lighting period (lights on from 07 : 00 to 19 : 00). Rats were fed a commercial diet (CLEA Japan, Incorporated, Tokyo, Japan) with tap water *ad libitum* for 1 week.

5. Experimental procedures

(1) Experiment 1 : ingestion of the persimmon fruit before alcohol administration

1) Human subjects : The age range of the recruited subjects was 20 ~ 50 years. A study population of 10 individuals (7 males and 3 females) who showed homo - or heterologous expression of the acetaldehyde - dehydrogenase 2 (ALDH2) gene were selected from 20 healthy adults by the results of the alcohol patch test[18] and the score of Tokyo University ALDH2 Phenotype Screening Test (TAST) [19].

This experiment was undertaken using fresh mature (in October), semi-dried persimmon and fresh apple fruit (in February). Each subject was fasted for 10 h before the experiment. At the end of fasting, the first baseline blood sample (5 mℓ / subject) was taken from each subject (0 h).

In control subjects, each volunteer drank a 90-mℓ serving of the Japanese alcoholic beverage sake (containing 15~16% or 11.0 g ethanol) over a 15-min period. There was an interval of 5 ~ 7 days between conduction of the control experiment and treatment experiment because the same subject could not repeat alcohol consumption in a short period. In the treatment group, each subject ingested either a single ripe fresh persimmon fruit or two semi-dried persimmon fruits or one fresh apple fruit 30 min before consuming sake, the same way as in the control subjects.

The second, third, and fourth samples of blood in the control and treatment subject were taken at 1, 2, and 3 h after alcohol administration, respectively.

2) Rats : Male rats (age, 24 weeks ; 380~440 g) were randomly divided into two groups of five after overnight fasting. The control group received vehicle (distilled water) only, whereas each rat in the test group received a dose of 10 mℓ / kg of 10% persimmon extract. Persimmon extract was administered orally using a gastric tube (Magensonde) before 30 min of alcohol loading. Both groups were given alcohol (2.0 g /kg). Baseline blood samples were collected before alcohol administration (0 h) and test samples were collected at 1, 2, 4 and 6 h after alcohol loading.

(2) Experiment 2 : ingestion of persimmon fruit after alcohol administration

1) Human subjects : A study population of 16 individuals (11 males and 5 females) was chosen from 33 healthy adults using the subject test selection methods described above. The ratio of the sex of subjects (male : female = 2 : 1) was designed to be consistent with that in experiment 1 (i.e., ingestion of the persimmon fruit was before alcohol administration). This experiment was undertaken using persimmon extract of the same persimmon cultivar from the fruit used in the earlier experiments. Each subject fasted for 10 h before the experiment. At the end of fasting, baseline blood samples (5mℓ/subject) were taken from each subject (0 h). After drawing the blood sample, each subject immediately drank a 70-mℓ serving of alcohol ('shochu', containing 25% or 14.3 g ethanol) over a 15-min period. Persimmon extract was not given after alcohol consumption for the control experiments.

After a 2-day interval, each subject repeated the experiment except that, 20 min after alcohol consumption, they drank a 35-mℓ persimmon extract containing approximately the same concentration of soluble polyphenols found in the whole fruit. The second, third, and fourth blood samples were taken at 1, 2, and 3 h after alcohol administration, respectively.

(3) Ethanol concentration in blood Whole blood (0.5mℓ) was added to a 5.0-mℓ screw-cap vial (SY-2 ; Nichiden-Rika Glass, Kobe, Japan). The vial was immediately sealed with a silicone-septum cap,

placed in an incubator at 27℃ for 25 min, and heated at 40℃ for 30 min in a water-bath. The 25-G needle of a glass syringe (volume, 1㎖) was then inserted into the septum. A 0.5-㎖ volume of the headspace vapor was drawn into the syringe and rapidly injected into the GC port at 210℃.

(4) GC condition The GC analysis was carried out on a GC 14 A series gas chromatograph with flame ionization detector (FID) (Shimadzu, Kyoto, Japan). The GC conditions were as follows: column temperature, 90℃ (the retention time of ethanol was ~10.2 min) or 90℃ to 150℃ (the retention time of ethanol was~9.4 min; 7 min hold at 90℃, 30℃/min from 90℃ to 150℃, and 8 min hold at 150℃); injection temperature, 210℃; detection temperature, 210℃; and helium flow rate, 60 ㎖/min. A chromosorb 101 (80/100 mesh) pre-conditioned glass column (1.6 m×3.2 mm I.D.; Shimadzu) was used. For GC quantification, the peak area of each compound was used. Ethanol was quantified with a calibration curve fit using 0.0~400 ppm (326㎍/㎖) ethanol solutions prepared in whole-blood samples from each subject.

(5) Experiment 3: flavonoids levels in the plasma of rats administered persimmon extract

1) Sampling method of the blood for detection of the absorption of kaki-tannin from the gastrointestinal epithelium to the blood vessels: Each rat (age, 5 weeks; 120~140 g) was fasted for 16 h before the experiments were started. At the end of fasting, a baseline blood sample (3㎖/rat) was taken from each rat (0 h), and each rat was immediately given persimmon extract (1㎖/rat) using a gastric tube (Magensonde). The second and third blood samples were taken at 2 h and 4 h after administration, respectively.

2) FRAP assay: We carried out the FRAP assay according to the methods of LOTITO and FREI[20]. In this assay, the antioxidant capacity of plasma was measured as ferric-reducing antioxidant potential using sampled blood.

3) Extraction and determination of flavonoids from plasma: We extracted the flavonoids from the plasma of rats administered persimmon extract according to the methods of SESINK *et al.*[21]. The extract product (20 ㎕) was analyzed by high-performance liquid chromatography (HPLC). The HPLC system (LaChrom, Hitachi, Tokyo, Japan) was fitted with an ODS-80 Ts column (4.6×250㎜). The system conditions were as follows: solvent was varied from 0 min to 10 min with acetonitrile/0.1% formic acid (10 : 90) followed by 10~20 min with a linear gradient of acetonitrile/0.1% formic acid (10 : 90) to acetonitrile/0.1% formic acid (30 : 70) and 20~50 min with acetonitrile/0.1% formic acid (30 : 70); flow rate, 1㎖/min; and electron capture detector (ECD) detection was set at 800 mV.

(6) Experiment 4: changes in soluble polyphenol content in persimmon fruit after incubation in artificial stomach liquid Persimmon flesh was cut into fine pieces, and ground into paste in a mortar and pestle. Four grams of the fruit paste with 20㎖ of artificial stomach liquid or distilled water was added to a 50-㎖centrifuge tube, and the tube was shaken. The centrifuge tube was incubated in an ultrasonic automatic washer at 37℃ for 5 min. After further incubation (without an ultrasonic automatic washer) at 37℃ for 0.5, 1, 2, and 3 h, the solution was centrifuged at 10,000 rpm for 30 min at 4℃.

The content of total polyphenols in the supernatant was measured by the Folin-Denis method as described by SWAIN and EILLIS[22]. Briefly, 1㎖ of sample (200 ㎕ of supernatant diluted with 800 ㎕ of distilled water) was made up to 7㎖ with distilled water, and mixed with 0.5㎖ of Folin-Denis reagent. After 3 min, 1㎖ of saturated Na_2CO_3 solution was added. The mixture was made up to 10㎖ with distilled water and left at room temperature for 60 min. The absorbance was measured with a spectrophotometer (Hitachi U-1100) at 725 nm. The determination was carried out thrice. The amount of phenolics (expressed as mg catechin/100 g fresh weight) was calculated from a standard curve (0.01~0.1㎎ catechin/㎖) prepared at the same time.

(7) Experiment 5: determination of the adsorption effect of kaki-tannin on ethanol gas/liquid

First, purified kaki-tannin (powder; 100 mg) was sealed in a 6-㎖ glass vial with an open-top screwcap and Teflon/silicon disk (Nippon Electric Glass, Shiga, Japan). In the control experiment, kaki-tannin was not added. Half a milliliter of saturated ethanol gas or 2㎖ of ethanol (99.5%) liquid was added to the sample or control 6-㎖ vial. The vial was shaken well to ensure mixing. Saturated ethanol gas was prepared as follows. Four milliliters of ethanol (99.5%) solution was sealed in a 19-㎖ vial and incubated at 35℃ for 20 min: the headspace gas was assumed to be saturated ethanol gas.

The ethanol gas concentration and amount of ethanol in ethanol solution in the vials were determined 2 h after incubation. Headspace gas (0.2. mℓ) or 1 μℓ of the solution was injected into the gas chromatograph. The GC condition was identical to that described in Experiment 2 except that the column, injection and detector temperatures were 160℃, 200℃, and 200℃, respectively.

6. Statistical analyses

The mean ± SE value from experiments was determined. Time-dependent changes in the concentration of ethanol in human blood between control and fresh persimmon or persimmon extract groups was evaluated by two-factor analysis of variance (ANOVA) with only one observation in each cell ($P < 0.05$). Among the controls, semi-dried persimmon and apple, pairwise comparisons were made using the Ryan-Einot-Gabriel-Welsch Q test ($P < 0.05$). Time-dependent changes in the ethanol concentration in rat blood between control and persimmon extract groups were evaluated by one-factor ANOVA ($P < 0.05$). A multiple comparison method for mean values of changes in the FRAP assay in rat plasma among different time points was undertaken using the Tukey-Kramer test ($P < 0.05$).

Results

1. Ingestion of the persimmon fruit before alcohol consumption

(1) Human subjects Among the data of 10 subjects, we excluded outlier values from 3 subjects. The mean values of the remaining 7 subjects who had a similar tendency with respect to changes in blood ethanol level were calculated and statistical analyses undertaken.

In the control group, the ethanol concentration in whole blood (n = 7) peaked 1 h after alcohol administration and returned to baseline level at 3 h. Blood ethanol levels in the fresh and semi-dried persimmon and fresh apple almost returned to the baseline level by 2 h (Fig.1).

1) Baseline results before alcohol consumption: The subjects in each group had whole-blood ethanol concentrations of~0mg/ℓ.

2) One hour after alcohol consumption: Fig.1-A shows that blood ethanol levels were lower in subjects treated with fresh persimmon (101.85 ± 29.89mg/ℓ) than those in the control (184.16 ± 29.27 mg/ℓ), and that this difference was significant at the 1% level. Fig.1-B shows that the blood ethanol levels were lowest in the semi-dried persimmon (96.37 ± 15.74mg/ℓ), and also that levels in the fresh apple (133.11 ± 14.33mg/ℓ) were lower than those in the control group (174.75 ± 13.95mg/ℓ). There were significant differences among each treatment and control at the 5% level.

3) Two hours after alcohol consumption: In the fresh persimmon group, the ethanol concentrations were lower (8.57 ± 8.57mg/ℓ) than in the control group (61.11 ± 28.99 mg/ℓ), but there was no significant difference at the 5% level (Fig.1 A). In the semi-dried persimmon (16.38 ± 12.38mg/ℓ) and fresh apple (17.58 ± 14.69 mg/ℓ), the ethanol concentrations were almost identical with those in the control group (29.44 ± 19.15mg/ℓ) (Fig.1 B).

4) Three hours after alcohol consumption: The mean whole-blood ethanol concentration of

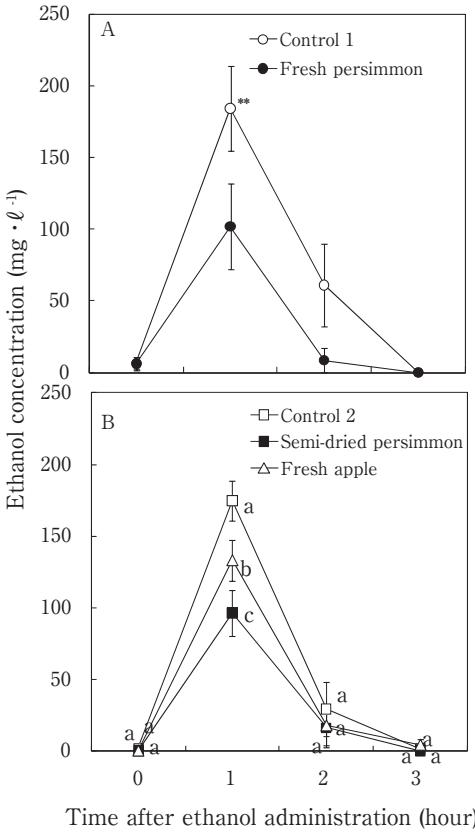

Fig. 1 Time-course of the whole-blood ethanol level in 7 human subjects who ingested fresh persimmon (A), or semi-dried persimmon fruit and fresh apple (B) before alcohol consumption

Each value is expressed as the mean of 7 replications ± SE. A: ** significant at $P < 0.01$ (two-factor ANOVA); B: pair-wise comparisons were made using the Ryan-Einot-Gabriel-Welsch Q test, different letters annotating values within each hour indicate significance at $P < 0.05$ among treatments and control.

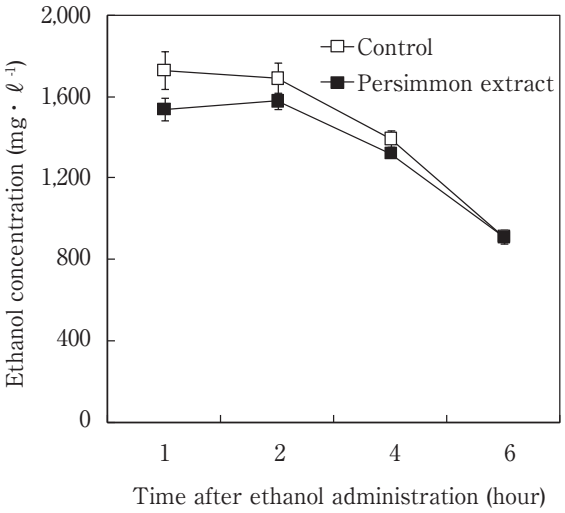

Fig. 2 Time-course of the whole-blood ethanol level in male rats who ingested persimmon extract before alcohol consumption

Each value is expressed as the mean of five replications ± SE. Significant differences between treatment and control were tested by one-factor ANOVA at the $P < 0.05$ level. Each value was not significant.

subjects in each group returned to baseline levels (Fig.1).

(2) Rats Blood ethanol concentrations measured at appropriate intervals after administered alcohol are shown in Fig. 2. The peak blood level was reached between 1 h and 2 h after alcohol administration. The persimmon extract group showed lower blood ethanol concentrations 1, 2 and 4 h compared with the control group, though the difference was not statistically significant.

2. Ingestion of persimmon extract after alcohol consumption in human subjects

There was no statistical significance at the 5% level in changes in whole - blood ethanol concentrations (n = 16) between persimmon (persimmon extract)-treated groups and control groups 1～3 h after alcohol consumption. However, the results suggested that blood ethanol levels in the treatment group were even higher than those in the control groups 1 h and 2 h after alcohol consumption (Fig.3).

3. Flavonoid levels in the plasma of rats after administration of persimmon extract

In the plasma of rats given persimmon extract, kaki-tannin consisting of four types of catechins was not detectable by HPLC (data not shown), and an increase in FRAP was not seen. However, there was a slight decrease in antioxidant activity over 4 h (Fig.4).

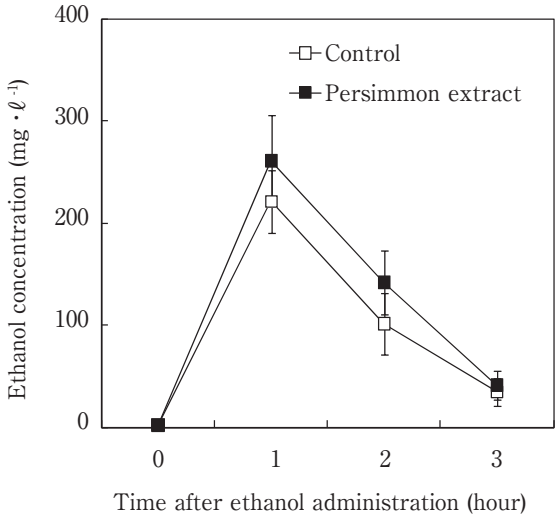

Fig. 3 Time-course of whole-blood ethanol levels in human subjects who ingested persimmon extract after alcohol consumption

Each value is expressed as the mean of 16 replications ± SE. Significant differences between treatment and control were tested by two-factor ANOVA at the $P < 0.05$ level. Each value was not significant.

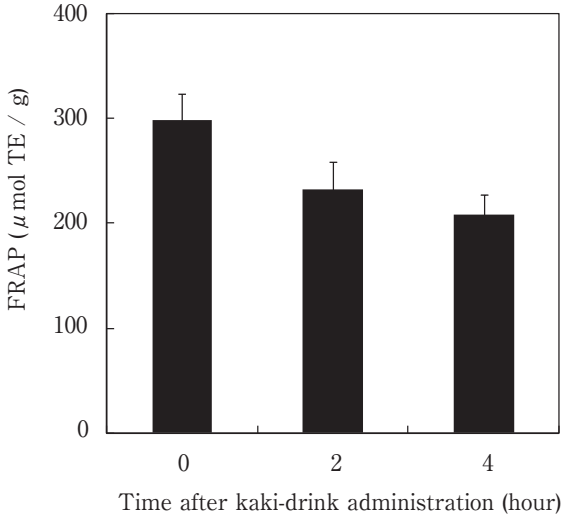

Fig. 4 Time-course of plasma antioxidant capacity in male rats after consumption of persimmon extract

Each value is expressed as the mean of 2～5 replications ± SE. Statistical analyses were undertaken by one-factor ANOVA at the $P < 0.05$ level. Significant differences among respective time periods were tested by the Tukey-Kramer *post-hoc* analysis. Each value was not significant.

4. Changes in soluble polyphenol content in persimmon fruit after incubation in artificial stomach liquid

There was a larger content of soluble polyphenols in 'Saijo' persimmon extract when it was incubated with artificial stomach liquid than when it was incubated in distilled water. The polyphenol content being incubated with artificial stomach liquid was significantly larger than that when it was incubated with water at 3 h (Fig.5).

5. Adsorption effect of kaki-tannin on ethanol gas /liquid

The level of ethanol gas (0.03mg/mℓ) after 2-h incubation with kaki-tannin was significantly lower than that in the control (1.5mg/mℓ) (Fig.6 A). Ethanol liquid after 2-h incubation with kaki-tannin showed no significant difference in ethanol amount compared with that in the control (Fig.6 B).

Discussion

In the present study, we studied the effects of 'Saijo' persimmon in lowering blood ethanol levels after alcohol consumption in humans. The time of ingestion of persimmon fruit, either before or after alcohol consumption, is an important factor in decreasing the ethanol concentration in blood. The effects of different times of fruit ingestion were also distinguished in this work.

Subjects who ingested fresh persimmon fruit before the consumption of alcohol showed a decrease in blood ethanol levels when compared with their corresponding control levels in which they did not receive fruit before drinking sake alcohol. One hour after alcohol consumption, the decrease in blood ethanol levels was significant (Fig. 1 A). The results for the semi-dried persimmon were nearly identical to those for the fresh persimmon (Fig.1 B). We also observed a similar (but not significant trend) in adult rats (Fig.2). Although there was a tendency of lowering blood ethanol levels by administration of persimmon extract in rats, this effect was not significant at the 5% level. This might have been due to the excessively high administration of alcohol, which was almost tenfold that used in humans.

These results that ingestion of persimmon before alcohol consumption can reduce blood ethanol levels are in accordance with those obtained from adult rabbits in the study of OGATA in which the fruit juice and peel of the 'Fuyu' (sweet type) fruit was used; blood ethanol levels were reduced markedly only if fruit juice or peel had been administered 30 min before alcohol administration[14]. Conversely, subjects who ingested persimmon extract after consuming alcohol had blood ethanol levels nearly identical to the controls at each time point after alcohol consumption (Fig.3). These results show that the whole-blood ethanol concentrations of subjects were affected only by the ingestion of persimmon fruit before alcohol consumption.

These results suggest that the ingestion of

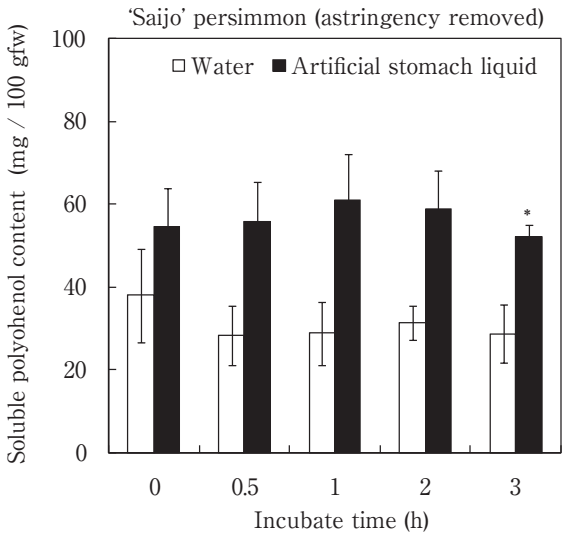

Fig. 5 Time-course of soluble polyphenol content in Saijo persimmon fruit after incubation in distilled water and artificial stomach liquid (pH 2) at 37℃

Each value is expressed as the mean of three replications ± SE. *significant at $P<0.05$ (one-factor ANOVA).

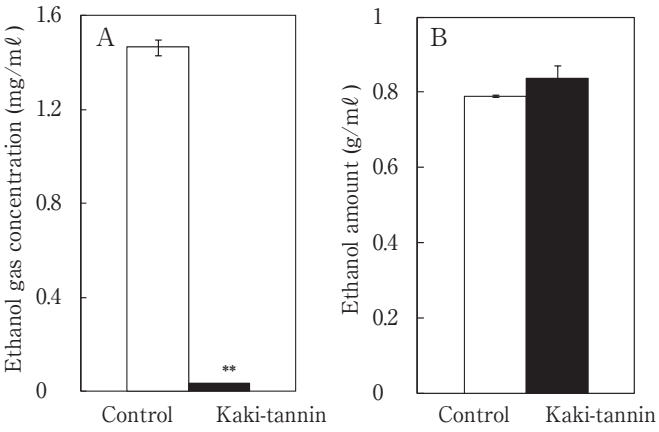

Fig. 6 Ethanol gas concentration (A) or ethanol amount in liquid (B) 2 h after incubation with kaki-tannin at room temperature compared with that in the control

Each value is expressed as the mean of three replications ± SE.
**significant at $P<0.01$ (one-factor ANOVA) between kaki-tannin treatment and control.

persimmon fruit before alcohol consumption may alleviate the deconditioning seen after excessive intoxication in humans. KOIKEDA, *et al*[15]. also reported that taking 'Saijo' persimmon extract before drinking alcohol could lower the ethanol concentration in human blood. This is an important finding, particularly for subjects who have low tolerance for alcohol, because the reduction in ethanol concentration was significant compared with control values.

However, it remains unclear which components of the persimmon fruit are involved in decreasing blood ethanol levels. According to our *in vitro* study, we showed that kaki-tannin (water-soluble) was extremely efficient in adsorbing ethanol gas (Fig.6 A), but it could not adsorb ethanol liquid (Fig.6 B). It is therefore likely that the highly functional compound kaki-tannin is related to the reduction of ethanol gas but not ethanol liquid in the human stomach. Usually, astringent-type persimmon fruit contains kaki-tannin in an exclusively soluble form (1.5 g/100 gfw [fresh weight]). After the removal of astringents in order to be edible, nearly all of the soluble tannin (high-molecular-weight) condenses to a higher-molecular-weight, insoluble tannin. Insoluble kaki-tannin is then ingested in the stomach, and part of it (15mg/100 gfw) is de-polymerized to soluble tannin under acid conditions, which was proved by our artificial stomach liquid experiment (Fig.5). Kaki-tannin was not absorbed from the surface of the gastrointestinal epithelium to the blood capillaries in our rat experiments (Fig.4), so we do not think that it plays a part in the ethanol concentration-decreasing effect in the blood and other organs (e.g., liver). Thus, we hypothesize that inside the stomach these de-polymerized soluble kaki-tannins (15mg/100 gfw) with the initial low-molecular-weight polyphenols (30mg/100 gfw; total, 45mg/100 gfw), as well as some dietary fibers (or probably in addition with the insoluble-type tannin) form a type of "film" to adsorb the ethanol gas which is released from the ingested ethanol liquid. This results in the disturbance of total ethanol absorption by the human digestive system (Fig.7). OGATA[14] also proposed that the intoxication-alleviating effect of the persimmon fruit was dependent upon depression of the absorption coefficient of ethanol on the surface of the gastrointestinal epithelium in the presence of persimmon fruit juice. OGATA's hypothesis supports our hypothesis to a certain extent.

Based on the above hypothesis, we might explain the different effects of persimmon and apple in decreasing blood ethanol levels (Fig.1 B). These might be due to the complete deficiency of soluble

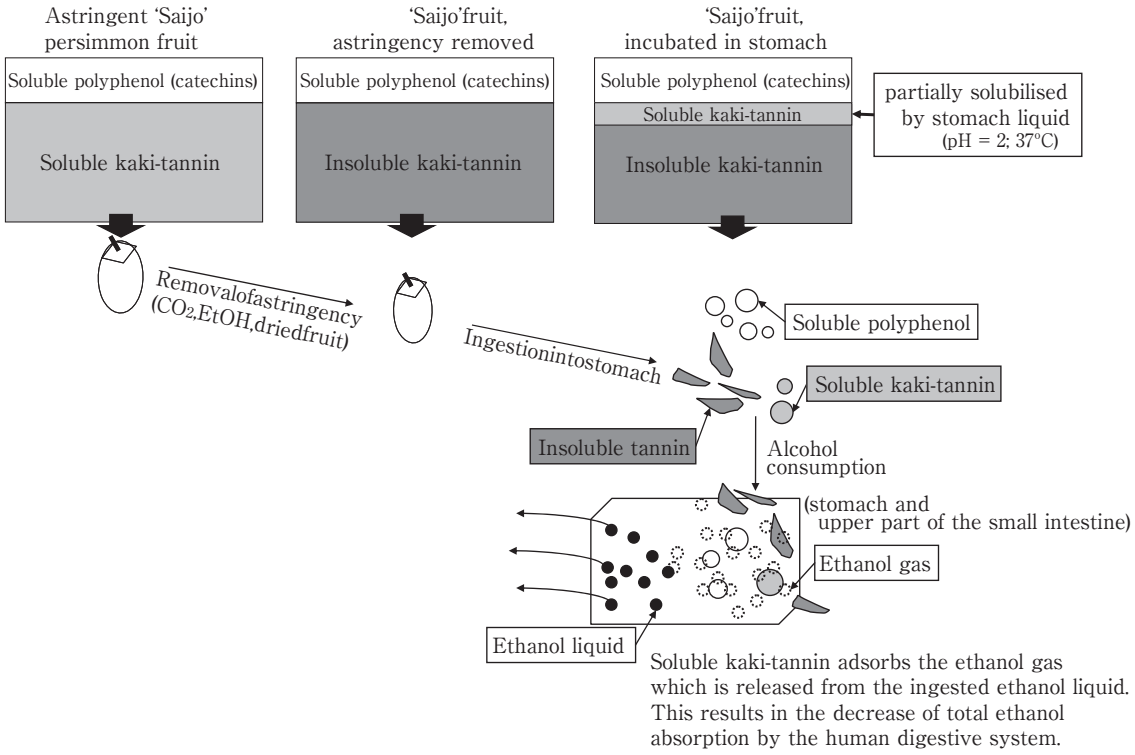

Fig. 7 Interactions of soluble kaki-tannin and ethanol in the human digestive system with respect to reduction in blood ethanol levels after alcohol consumption (schematic)

Table 1 Dietary fibers and tannin (polyphenol) contents in persimmons and apples (g/100 gw)

		Dietary fibers[z]		Tannin[y]		Total polyphenols[x]
		Soluble	Insoluble	Soluble	Insoluble	
Japanese Persimmons	Astringent, raw	0.5	2.3	1.5～2.0	Tr[w]	1.51[v]
	Astringency removed, raw	0.5	2.3	Tr	1.5	1.51
	Dried	1.3	12.7	Tr	2.2～4.2	7.48
Apples	Raw	0.3	1.2	0	0	1.2～2.4[u]

[z] Data for persimmons and apples are cited from Tables of Food Composition 2009 (Fifth Revised and Enlarged Edition) 2009[23].
[y] Data for persimmons are cited from study of TAIRA *et al*., 1992[24], 1998[25], and 2008[26].
[x] In persimmon, total polyphenols contain kaki-tannin and other catechins; In apple, total polyphenol usually includes variety of soluble flavonoids and other phytochemicals.
[w] Tr means the content is trace level.
[v] Data for persimmons are cited from study of JUNG *et al*., 2005[6].
[u] Data for apples are cited from study of BOYER and LIU, 2004[27].

kaki-tannin, or both soluble and insoluble tannin in apple. Also, the contents of dietary fibers in apple fruit are lower than that of persimmons (Table 1). Both of these aspects might contribute to the higher efficiency in ethanol alleviation in persimmon.

Future experiments (including those with animals) will be essential for resolving the mechanisms by which the persimmon fruit decreases blood ethanol levels after alcohol consumption in humans.

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飲酒前後のカキ‘西条’果実摂取がヒト血中のエタノール濃度に及ぼす影響

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渋ガキ‘西条’果実は，酩酊したヒトの体調の悪化を緩和できることが知られている。本研究では，カキを摂取することで飲酒後血中エタノール濃度の低下に寄与できるかどうかを評価するために，ヒトを対象に飲酒前後にカキ（カキ果実，あんぽガキまたはカキ抽出液）を摂取したときの血中エタノール濃度を調査するとともに，飲酒前のリンゴ摂取との比較も行った。また，ラットに対して，アルコール摂取前にカキ抽出液を投与して血中濃度を測定した。さらに，カキ抽出液を投与したラットの血漿中の抗酸化力（鉄還元能FRAP値）の変化とフラボノイドの有無を調べた。その結果，ヒトへの飲酒前のカキ摂取において，血中エタノール濃度はアルコール摂取後１時間で対照およびリンゴ摂取よりも有意に低くなった。さらに，アルコール摂取前にカキ抽出液を投与したラットについても，有意差はないものの同様の傾向が認められた。一方，飲酒後にカキを摂取した被験者の血中エタノール濃度は，対照と比べて有意差が認められなかった。カキ抽出液を投与したラットの血漿中にはフラボノイドは検出されず，FRAP値の増加も認められず，むしろFRAP値は減少する傾向さえみられた。これらのことから，本研究は，飲酒前のヒトに，カキ果実中に含まれる機能性成分であるカキタンニンを，単独もしくは他の成分とともに摂取させることによって，血中エタノール濃度を低下させる効果があることを示した。カキの機能性成分は血中に移行しないため，これらはヒトの胃の中でエタノールを吸収し，胃腸の上皮表面からのエタノールの吸収を抑制するものと考えられる。

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A Comparative Study of the Effects of Postharvest Treatments on Occurrence of Kohansho and Quality of 'Kiyomi' Tangor

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The effects of postharvest treatments, namely, perforated film packaging (PFP), hot water dip (HWD), and hot calcium dip (HCD), on the occurrence of "Kohansho (rind disorder)" and the quality attributes of 'Kiyomi' Tangor (*Citrus unshiu* Marc. ×*C. sinensis* Osb.) fruit were compared. Samples of the fruit were treated, packed in corrugated fiberboard boxes, and stored at 10℃ for up to 4 weeks. Weight loss was significantly lower and firmness was significantly higher in the fruit treated by PFP than in those treated by other postharvest treatments or in the control fruit. All postharvest treatments reduced the occurrence of Kohansho compared with control, and PFP and HWD treatments were more effective than HCD in reducing the occurrence of Kohansho. None of the postharvest treatments impaired quality attributes tested (total soluble solids [TSS], titratable acidity [TA], TSS/TA ratio, ascorbic acid content, and ethanol content), when compared with the attributes of the control. We conclude that PFP treatment, which can maintain a high relative humidity and a modified atmosphere except in the presence of high CO_2 levels (＞8％) and/or low O_2 levels (＜5％) was the most effective and commercially practical method to control Kohansho and preserve quality.
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Key words : *postharvest treatments, perforated film packaging, hot water dip, kohansho, 'Kiyomi' Tangor*
収穫後処理，有孔フィルム包装，温湯浸漬，こはん症，タンゴール清見

'Kiyomi' Tangor (*Citrus unshiu* Marc. ×*C. sinensis* Osb.) is recognized as a high-quality late-season citrus fruit. Unfortunately, this variety is vulnerable to Kohansho, a serious, non-chilling physiological rind disorder that lowers quality during storage, transportation, and marketing periods. Kohansho initially appears as small pitting, later developing into a brown sunken area. The disordered fruit is usually rejected by the market.

Individual seal -packaging reduces the occurrence of Kohansho[1], and storage under low temperature and high relative humidity conditions without prestorage conditioning reduces the occurrence of rind injury in 'Kiyomi' fruit[2]. In our previous studies, we observed that Kohansho can be suppressed by reducing fruit weight loss during storage[3], and it was recommended that in order to prevent Kohansho and preserve high quality, 'Kiyomi' fruit should be stored under modified atmosphere conditions, with high relative humidity, and should not be exposed to high CO_2 levels (＞8％) and/or low O_2 levels (＜5％)[4].

Hot water dip (HWD) treatment (50℃ for 3～5 min) reduced the occurrence of Kohansho in Navel orange[5], Natsudaidai[5] and Hassaku[5,6]. Similar HWD treatment (50～53℃ for 2～3 min) reduced the incidence of chilling injury in 'Star Ruby' grapefruit[7] and various cultivars of blood oranges[8] without impairing quality. Calcium dip treatment was reported to lessen the damage due to many physiological disorders and preserve fruit quality by maintaining firmness and reducing weight loss and decay in a variety of fresh fruits and vegetables[9]. A combined calcium and hot water (hot calcium dip, HCD) treatment[10] is therefore expected to be more effective than either HWD or calcium dip treatment

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alone.

However, there is no information about the effect of HWD and HCD treatment on 'Kiyomi' Tangor. The aim of the present study was to compare the effects of perforated film packaging (PFP), HWD, and HCD treatments on the occurrence of Kohansho and the quality of 'Kiyomi' Tangor fruit.

Materials and Methods

1. Plant material

'Kiyomi' Tangor fruit (diameter, 7.3～8.0 cm) harvested on March 19, 2008, from Misaki, Ehime, were transported to the Laboratory of Agricultural Process Engineering, Ehime University. The fruit without Kohansho were used for the experiment on the following day (March 26, 2008).

2. Postharvest treatments

The fruit were treated with different postharvest treatments as follows.

(1) PFP treatment The fruit were sealed in a perforated low-density polyethylene film package (a 0.019-mm film of dimensions 60 × 50 cm, with 15 perforations, each 5mm in diameter) and packed in a corrugated fiberboard box (dimensions 36 × 25 × 15 cm). This perforated package was designed in accordance with the modified atmospheric conditions recommended by TECHAVISES and HIKIDA[4].

(2) HWD treatment The fruit were dipped in a hot water bath at 53℃ for 2 min, dried at room temperature for about 2 h, and packed in a corrugated box. The temperature in the hot water bath was maintained at 53 ± 0.5℃ using an electronic thermostat.

(3) HCD treatment The fruit were dipped in hot 1% (w/v) calcium chloride solution at 53℃ for 2 min, dried at room temperature for about 2 h, and packed in a corrugated box.

(4) Control The fruit were packed in a corrugated box without treatment.

Three replicates of 5kg of 'Kiyomi' fruit (about 28 fruits) were used in each treatment. All corrugated boxes were stored at 10℃ for up to 4 weeks. The temperature and relative humidity (RH) inside the boxes, inside the packages in the PFP treatment, and inside the storage chamber were monitored throughout the storage period by using temperature and humidity sensors. The vapor pressure deficit (VPD) between the fruit surface and the surrounding air, a major driving force of transpiration, was calculated by subtracting the vapor pressure of the surrounding air from 98% of saturated vapor pressure at the surrounding air temperature.

3. Evaluation of Kohansho

Three replicates of 16 fruits in each treatment were checked for Kohansho by visual inspection at 1－week intervals. The number of fruit and the diameter of affected areas were measured and rated using the following index : 0 = no occurrence of Kohansho ; 1 = one spot of less than 5 ㎜ ; 2 = one spot of 6～20 ㎜ ; 3 = 2～3 spots of 20 ㎜ ; 4 = more than 4 spots of 20 ㎜.

The results were expressed as the percentage and degree of Kohansho :

$$\text{Percentage of Kohansho}(\%) = \frac{\text{total number of fruit incurring Kohansho}}{\text{total number of fruit}} \times 100$$

$$\text{Degree of Kohansho} = \frac{\Sigma(\text{Kohansho index} \times \text{number of fruit incurring Kohansho in each index})}{4 \times \text{total number of fruit}} \times 100$$

4. Determination of quality attributes

Three replicates of 16 fruits in each treatment (the same samples that were used to evaluate Kohansho) were weighed at 1-week intervals to assess percentage weight loss. The firmness, and the content of total soluble solids (TSS), titratable acidity (TA), ascorbic acid (AA), and ethanol were determined in 3 replicates of 4 fruits each, on day 0 (fresh) and day 28 (end of storage). Fruit firmness was determined with a fruit hardness tester using a 1-kg weight. Juice TSS content was measured with a digital refractometer, juice TA was measured by titration with 0.1 M NaOH, and the results were expressed as citric acid percentages. Juice AA content was determined by the indophenol titration method[11].

Ethanol, the dominant fermentative metabolite, can be used to indicate fermentative metabolism for mandarin fruit[12]. The acetaldehyde and ethanol that accumulate during fermentation can lead to the development of off-flavors, thus impairing fruit quality[13]. Juice ethanol content was determined by incubation of 10-mℓ aliquots of juice in 50-mℓ glass vials sealed with an aluminum cap at 30℃ for 30 min following SCHIRRA *et al.*[8]. Aliquots of 10-mℓ each of ethanol standards of 1,000, 2,000, 3,000, 4,000, and 5,000 (μℓ / ℓ) were used to generate a calibration curve. After incubation, a 1-mℓ gas sample was withdrawn from the headspace and analyzed with a gas chromatograph (GC ; model GC-8 A,

Shimadzu, Japan) coupled with an FID and a stainless steel column (length and internal diameter, 200 × 0.2 ㎝) containing 15% Carbowax and Chromosorb W 80/100 mesh. The temperatures of the injector, column, and detector were 100, 90, and 100℃, respectively, and nitrogen was used as the carrier gas.

5. Atmosphere analysis

A gas sample of 0.5㎖ was taken from inside each PFP package and from inside the storage chamber at 3-day intervals. O_2 and CO_2 concentrations were determined using a GC (model GC-8 A; Shimadzu, Japan) coupled with a TCD and a stainless steel column (length and internal diameter, 180 cm×0.6 cm) containing WG-100. The temperatures of the injector, column, and detector were 80, 50, and 80℃, respectively. Helium was used as the carrier gas.

6. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) by the SPSS software (Windows version 11.5; SPSS Inc., Chicago, IL, USA). The significance of differences between means was determined by the Tukey HSD test ($P<0.05$).

Results and Discussion

The 'Kiyomi' Tangor fruit in all treatments lost weight during storage; the weight loss was significantly lower in the PFP treatment than in other treatments and control (Table 1). This was because the notably higher RH led to lower vapor pressure deficit (VPD) in the PFP treatment than in the other postharvest treatments or control (Table 2). VPD is a major driving force for weight loss, since higher VPD results in higher transpiration.

The fruit that underwent all the postharvest treatments showed lower percentage and degree of Kohansho than the control fruit, but the PFP and HWD treatments were more effective than the HCD treatment on reducing the occurrence of Kohansho (Table 3). In the PFP treatment, the average O_2 and CO_2 concentrations inside the package during the storage period were 20.40% and 0.97%, a slight change from normal atmospheric level ($P<0.05$). According to our previous study[4], such a slight change does not affect the occurrence of Kohansho. Therefore, the effectiveness of PFP treatment results from the generation of high RH

Table 1 Weight loss of 'Kiyomi' Tangor fruit during storage at 10℃ under different postharvest treatments

Treatment	% Weight loss			
	Week 1	Week 2	Week 3	Week 4
PFP	0.17±0.01 c	0.30±0.01 b	0.48±0.01 b	0.66±0.02 b
HWD	1.74±0.04 ab	2.95±0.06 a	4.40±0.08 a	5.89±0.11 a
HCD	1.82±0.04 a	3.09±0.07 a	4.61±0.10 a	6.13±0.13 a
Control	1.65±0.04 b	2.93±0.08 a	4.54±0.12 a	6.14±0.15 a

Data are represented as mean±S.E. of 3 replicates of 16 fruits each.
Column means followed by different letters are significantly different at $P<0.05$.

Table 2 Average temperature and RH inside the corrugated fiberboard box (HWD, HCD, control), inside the PFP package, and inside the storage chamber throughout the storage period, and the vapor pressure deficit (VPD), in each treatment

Treatment	Temperature (℃)	RH (%)	VPD* (kPa)
PFP	10.67	99.21	0.01
HWD	10.06	79.16	0.27
HCD	10.25	79.85	0.26
Control	9.82	82.35	0.22
Chamber	9.73	49.17	−

*VPD was calculated by subtracting the vapor pressure of the surrounding air from 98% of saturated vapor pressure at the surrounding air temperature.

Table 3 Effect of postharvest treatments on occurrence of Kohansho in 'Kiyomi' Tangor fruit during storage at10℃

Treatment	Percentage of Kohansho (%)				Degree of Kohansho			
	Week 1	Week 2	Week 3	Week 4	Week 1	Week 2	Week 3	Week 4
PFP	0.00	0.00	2.08	2.08	0.00	0.00	0.52	0.52
HWD	0.00	0.00	0.00	2.08	0.00	0.00	0.00	1.04
HCD	0.00	0.00	2.08	6.25	0.00	0.00	1.04	2.60
Control	2.08	2.08	4.17	14.58	0.52	0.52	1.56	5.73

Data are means of 3 replicates of 16 fruits.

surrounding the fruit; this result is consistent with our previous findings[3),4)]. The HWD treatment was as effective as the PFP treatment (Table 3). Similar effects of the HWD treatment have also been reported in Navel orange[5)], Natsudaidai[5)], and Hassaku[5),6),14)]. There was no significant difference in weight loss between the HWD and control groups; therefore, other mechanisms may act to limit Kohansho. For example, MAKITA[5)] reported that the HWD treatment of Navel oranges enhanced the reducing sugar content in the peel, which is associated with Kohansho tolerance. The fruit that underwent the HCD treatment also showed reduced occurrence of Kohansho compared with the control fruit but was not as effective as the PFP and HWD treatments (Table 3). This implies that calcium was not as effective as expected; in contrast, the calcium treatment reduced the effectiveness of HWD. MANAGO[6)] also reported increased occurrence of Kohansho in Hassaku after dipping in a 2% $CaCO_3$solution. These results suggest that the best results can be achieved by a combination of the PFP and HWD treatments.

Firmness was the highest in fresh fruit (determined on day 0) and decreased during storage in all treatments, but it was significantly higher in the PFP treatment group at the end of storage. This result is consistent with the observation that the weight loss was lower in the PFP treatment group than in the other treatment or control groups (Table 4). Our previous study[4)] found that use of modified atmospheric conditions maintains the firmness of 'Kiyomi' Tangor via the maintenance of a high RH inside the package. BEN-YEHOSHUA *et al*[15)]. also reported that the high humidity of the micro-atmosphere created inside modified atmosphere packaging maintains the turgidity of the tissue and markedly inhibits water loss and softening in citrus fruits.

The TSS content did not change, but the TA content tended to decrease, resulting in an increase in the TSS/TA ratio after 4 weeks of storage in all treatment and control groups, compared with the ratio in the fresh fruit (Table 4). The AA content decreased slightly and the ethanol content tended to increase in all the treatment and control groups compared with the contents in the fresh fruit (Table 4). Ethanol was seen to accumulate during the storage period even in the control group, consistent with our previous observation[4)]. However, there were no significant differences in TSS, TA, TSS/TA ratio, AA content, and ethanol content between any

Table 4 Effect of postharvest treatments on the quality attributes of 'Kiyomi' Tangor fruit

Treatment	Firmness (kg)	TSS (%)	TA (%)	TSS/TA ratio	AA (mg/100mℓ)	Ethanol* (μℓ / ℓ)
Fresh	0.922±0.005 a	12.4±0.4 a	1.09±0.05 a	11.6±0.6 a	58.3±1.8 a	1568±215 a
PFP	0.887±0.009 b	12.5±0.2 a	0.93±0.04 ab	13.6±0.6 a	48.7±1.6 b	1817±340 a
HWD	0.847±0.005 c	12.2±0.3 a	0.98±0.11 ab	15.6±3.2 a	51.1±1.4 b	2533±227 a
HCD	0.857±0.005 c	12.3±0.3 a	0.81±0.06 b	16.2±1.4 a	52.6±1.6 ab	2596±304 a
Control	0.833±0.006 c	11.9±0.5 a	0.79±0.05 b	15.4±0.9 a	50.9±1.7 b	2000±250 a

Data are represented as mean±S.E. of 3 replicates of 4 fruits each.
In the case of the 'fresh' condition, all parameters were determined on day 0 (initial day of experiment), while in the case of all other treatments, the parameters were determined on day 28 (end of storage).
Column means followed by different letters are significantly different at $P<0.05$.
*Ethanol is an indicator of fermentative metabolism.

of the postharvest treatment groups and the control group after 4 weeks of storage.

On the basis of the results obtained and considering the recommended modified atmosphere conditions[4], the PFP treatment, which maintains high RH and a modified atmosphere except in the presence of high CO_2 levels (＞8％) and/or low O_2 levels (＜5％), appears to be the most effective means to control Kohansho and preserve quality.

Conclusions

The results clearly showed that PFP is better than other postharvest treatments in reducing weight loss and incidence of Kohansho, as well as in retaining firmness and other quality attributes. Since Kohansho is caused mainly by fruit weight loss[3], we conclude that PFP treatment reduces Kohansho by restricting water loss. The HWD and HCD treatments reduce the occurrence of Kohansho even when the fruit lose weight, and these treatments are effective during storage, transportation, and marketing periods where some water loss is expected. Since the PFP treatment is more convenient and economical than the HWD or HCD treatment, the PFP which can maintain a high RH and a modified atmosphere except in the presence of high CO_2 levels (＞8％) and/or low O_2 levels (＜5％), may be preferable as a practical commercial method of Kohansho prevention and quality maintenance in 'Kiyomi' Tangor.

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タンゴール‘清見’果実のこはん症発生と品質に及ぼす収穫後処理の効果

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有孔フィルム包装 (PFP), 温湯浸漬 (HWD), 温湯カルシウム浸漬 (HCD) の収穫後処理が‘清見’果実のこはん症発生と品質に及ぼす効果を比較した。3種類の処理を施した果実を段ボール箱に収納し, 10℃で4週間貯蔵した。PFP処理区では, ほかの処理および対照区 (無処理) と比較して, 重量減少が明らかに小さく, 果実硬度が明らかに高い結果が得られた。すべての処理はこはん症の発生を減少させたが, PFPとHWD処理はHCD処理より高い減少効果を示した。また, これらの処理は対照区と比較して, 糖度 (TSS), 滴定酸度 (TA), TSS/TA比, アスコルビン酸含量, エタノール含量を損なうことはなかった。これらの結果より, 高湿度条件を保ち, かつ8％以上のCO_2濃度環境と5％以下のO_2濃度環境を避けたPFP処理はこはん症の抑制と品質保持において最も効果的で, 商業的に実用的な方法であると考えた。

Purification and Characterization of Wheat Protein Disulfide Isomerase Expressed in *Escherichia coli*

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A protein disulfide isomerase (PDI) coding sequence was cloned from wheat (*Triticum aestivum* cv *'Haruyutaka'*). The cDNA contained 3 distinct sequences; 2 of them, wPDI 1 and wPDI 3, were 1548 bp in length, and the other, wPDI 2, was 1539 bp in length. Sequences wPDI 1 and wPDI 2 shared 98.6% identity, wPDI 1 and wPDI 3 shared 99.2% identity, and wPDI 2 and wPDI 3 shared 99.0% identity. The total PDI activity of recombinant (r) PDI was 114 U, and the specific activity was 336 U/mg. The rPDI catalyzed the formation of disulfide bonds in disulfide reduced and denatured ovalbumin. The optimal temperature for the 2 PDIs (rPDI and native PDI) was 35℃, and PDI activity was highest at pH 8.5. Furthermore, the rPDI was superior to the native PDI with respect to heat and pH stability; however, the rPDI and native PDI had similar optimum conditions. This study is the first to demonstrate recombinant expression of wheat PDI.

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Key words: *protein disulfide isomerase, disulfide bonds, PDI*
プロテインジスルフィドイソメラーゼ, ジスルフィド結合, PDI

Protein disulfide isomerase (PDI 5.3.4.1) is an essential protein for the formation, reduction, or isomerization of protein disulfide bonds, depending on the conditions in the lumen of the endoplasmic reticulum (ER). PDI is a member of the thioredoxin superfamily and it contains 2 thioredoxin-like active sites (CGHC), which act as the catalytic sites for isomerase activity[1]. This family contains thioredoxin, thioredoxin-2[2], DsbA[3], etc. Thioredoxin, thioredoxin-2, and DsbA contain only thioredoxin active sites. Thioredoxin-2 is a mitochondria-type thioredoxin[2], whereas DsbA is localized to the periplasm of prokaryotes. The characteristics of plant PDI, the regulation of its expression during plant development, and its physiological role are not well known. Studies on plant PDI expression and intracellular localization in wheat and maize[4),5] have indicated that the enzyme may play an important role in the folding of plant secretory proteins, particularly in the formation of endosperm protein bodies. Plant PDI or PDI-like cDNA sequences have been cloned and sequenced from species such as alfalfa[6),7], barley[8], common wheat[9], maize[5], and castor bean[10]. Wheat PDI cDNA has been isolated from root tip, but this protein has not been recombinantly expressed or characterized. In this paper, we report the isolation and characterization of a cDNA encoding PDI from wheat and the expression of wheat PDI. Subsequently, we analyze the characteristics of the expressed recombinant PDI.

Materials and methods

1. Primers for PCR amplification

Primers for the amplification of PDI were synthesized based on the common wheat cv Chinese Spring[11] and cv Wyuna cDNA sequences[12]. Primers wPDI-F (5′-TGCTCCCCAGTYCCTTCCGCC-3′; bases 5－25 of AF 262979) and wPDI-R (5′-CTGGC-GTTGGAAGCTGCCTTGCCA-3′; bases 1623－1646 of AF 262979) were used for the amplification of PDI cDNA including the full-length open reading frame (ORF). To express precursor PDI (prePDI) cDNA using pET-21 a (Novagen, USA) in *Escherichia*

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coli, the forward primer wPDI-Nde I-F (5′-ATGCCATATGGCGATCTSCAAGG-YCTGGATC-TCGCT-3′; bases 26–54 of AF 262979), containing an *Nde*I site and the reverse primer wPDI-Xho I-R (5′-GCATCTCGAGGCAGAGCTCGTCCTTCAGAGGCTC-3′; bases 1549–1573 of AF 262979), containing an *Xho*I site were used.

2. Preparation of *T. aestivum* cv *Hruyutaka* cDNA

Dormant seeds were soaked in water for 15 min at room temperature. Young roots were harvested after cultivation for 2 days at 28℃. Harvested roots were immediately frozen in liquid nitrogen and used for the preparation of total RNA using the RNeasy plant mini kit (Qiagen, USA) according to the manufacturer's instructions. RNA concentrations were determined by measuring the absorbance at 260 nm. The isolated RNA was transcribed into cDNA using the PrimeSTAR RT-PCR Kit (TaKaRa, Japan), with thermal modification.

3. Cloning and Sequence analysis of PDI (*T. aestivum* cv *Hruyutaka*) and phylogenetic analysis of Poaceae

Amplification of PDI from cDNA was performed by PCR using PrimeSTAR HS DNA Polymerase (TaKaRa, Japan). The PCR was performed as follows: 35 cycles of 10 s at 98℃, 5 s at 60℃, and 120 s at 72℃. The amplified fragment was inserted into pUC 118 (TaKaRa, Japan), and was sequenced by the dideoxy chain-termination method[13] using a BigDyeTerminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The PDI sequences of *T. aestivum* and neighbor related taxa were obtained from DDBJ, and the distances were calculated according to the method of JUKES and CANTOR[14]. A phylogenic tree was constructed by the neighbor-joining method[15] using the CLUSTAL X program (version 1.8;[16]).

The 3-dimensional protein structure of each recombinant PDI was predicted based on the amino acid sequence using 3 D-JIGSAW (http://www.bmm.icnet.uk/servers/3djigsaw/) and Chimera software[17,18].

4. Overexpression and purification of recombinant PDI from *E.coli*.

MEADOWS and ROBINSON[19] previously reported that wheat protein precursors are expressed in *E. coli*, and that mature protein is produced in these cells. Therefore, for effective expression of wheat PDI in *E. coli*, the presence and location of signal peptide cleavage sites in prePDI were predicted using the gram-negative prokaryote database in the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/)[20] before expressing recombinant PDI. The result showed that the location of the signal peptide cleavage site corresponds to that of wheat, and computational analysis showed that this site was probably cleaved (99.9% probability) in *E. coli* (data not show). Therefore, we predicted that the recombinant PDI expressed from DNA encoding PDI, including the wheat PDI signal peptide (prePDI) would be nearly identical to native PDI.

For intracellular recombinant expression, the prePDI cDNA was inserted into the expression vector, pET 21 a (Fig. 1). The resulting expression plasmid, pET 21 a/prePDI, was used to transform *E. coli* BL 21 (DE 3) (Stratagene, USA). *E. coli* BL 21 (DE 3) + pET 21 a/prePDI was cultured in 100mℓ of LB broth containing ampicillin (100μg/mℓ) at 37℃, until the optical density at 600 nm was 0.6. Expression of rPDI was induced by incubation with IPTG (0.2 mM) for 16 hr at 20℃.

The cells were harvested by centrifugation at 5,000× g for 20 min at 4℃. The cell pellets were resuspended in 10mℓ of 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, and were disrupted by an

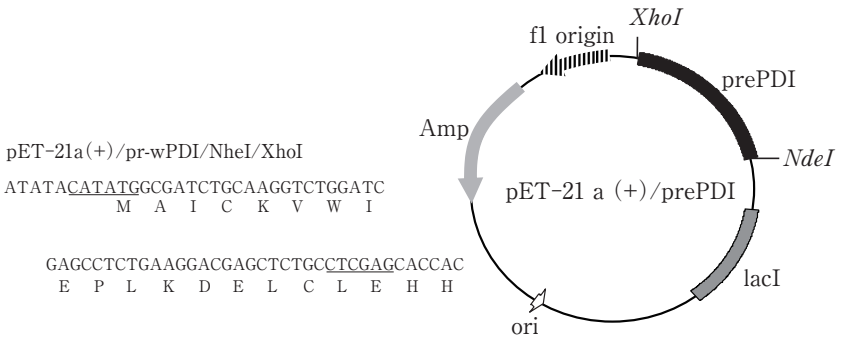

Fig. 1 Expression plasmid for wheat PDI

pET-21/prePDI: a plasmid containing the wheat prePDI cDNA including signal peptide inserted between the *Nde*I and *Xho*I sites

ultrasonic oscillator (US 150; Nihon Seiki Seisakusho, Japan). The cell debris was removed by centrifugation (140,000× g, 60 min, 4℃). Then, the clear supernatant was used for protein purification. A TALON metal affinity resin column (TaKaRa, Japan) was first equilibrated with 10 column volumes of start buffer (20 mM Tris-HCl, 300 mM NaCl, pH 8.0). Then the clarified sample was applied to the column. The column was washed with 10 column volumes of start buffer, and the protein was subsequently eluted with elution buffer (start buffer containing 40 mM imidazole). The protein content was determined by the Bradford method[21] using BSA as a standard.

5. Assay for PDI activity

The activity of PDI was measured using the method of Lundstrom and Holmgren[22),23)]. The incubation mixture contained 0.5 ㎎ / ㎖ insulin (TaKaRa, Japan), 0.1 M potassium phosphate (pH 8.0), 2 mM EDTA, and PDI protein extract (20 ㎕). The reaction was initiated by the addition of 132 ㎕ of 5 mM DTT in a final volume of 2 ㎖, after incubation for 40 min at 25℃. The increasing turbidity of insulin peptide chains was measured at 650 nm.

6. Amino acid sequence analysis and research on the cubic structure of proteins

The eluted fractions, cleared lysate, and total cell extract were analyzed using SDS-PAGE by the method of LAEMMLI[24] on a 10% (v/v) acrylamide gel. The gels were stained with Coomassie Brilliant Blue R-250 and G-250. The purified recombinant PDI, which was designated rPDI, was stored at 4℃ until analysis.

The N-terminal amino acid sequence of the 2 kinds of recombinant PDI were determined by automated Edman degradation using a protein sequencer (PPSQ-21; SHIMADZU, Japan)[25].

7. Assay for PDI folding activity (rPDI)

The PDI folding activity was measured by the method of KAWAMURA[26]. The reaction mixture contained 0.5㎎/㎖ disulfide reduced and denatured ovalbumin, 50 mM Tris-HCl (pH 8.2), 1 mM EDTA, and either 0.5 μM rPDI or no PDI (negative control). The prepared ovalbumin was refolded by PDI at 25℃ and samples were taken from the reaction after 0, 5, 10, and 15 hr of incubation. These reaction samples were diluted 10-fold with 50 mM Tris-HCl (pH 8.2) and 1 mM EDTA, then the far-UV CD spectrum of ovalbumin was recorded at 25℃ with a spectropolarimeter (J-720 WI; JASCO, Japan). The CD data were expressed as mean residue ellipticity (degrees · ㎠ · $dmol^{-1}$) using 111 as the mean residue weight of ovalbumin.

8. Two-dimensional gel electrophoresis and Western blot native PDI comparison with rPDI

Separation of proteins by first-dimensional IEF electrophoresis was performed using agar-GEL (pH 3–10, ATTO, Japan) according to the manufacturer's instructions[27]. Partially purified native PDI and purified rPDI samples dissolved in sample solvent buffer were loaded using a sample cup. IEF electrophoresis was performed at 300 V for 210 min.

Before carrying out the second-dimensional SDS-PAGE, the agar-GEL was entrenched and equilibrated. The equilibrated agar-GEL was transferred onto the SDS-PAGE, and then, SDS-PAGE was performed using the method of LAEMMLI[24].

The rPDI was electrophoresed and the gel was stained with CBB R-250 and G-250. Meanwhile, the electrophoresed gel of the partially purified native PDI was electroblotted onto a PVDF membrane and incubated with anti-wPDI antibody, which was detected with horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Japan)[28]. Bound antibodies were visualized using an ECL-Plus chemiluminescence system (GE Healthcare). Gel images were obtained using a gel image analyzer (Gel-Doc; Bio-Rad, USA). Antiserum was obtained by immunizing a mouse with rPDI antigen.

9. Influence and stability of pH and temperature on native PDI and rPDI

The relative activity was determined at various pH and temperatures. Experiments were carried out with the purified rPDI and the partially purified native PDI from wheat seed[29]. The influence of pH on PDI activity was determined at 25℃ by using the assay protocol described above. To test pH stability, rPDI was diluted to 1 μM (about 34 U), while the native PDI was diluted 2-fold using respective buffers with pHs ranging from 4 to 10, and then the rPDI and native PDI were incubated at 4℃ for 8 hr and 4 hr, respectively. The residual activity was determined using these enzymes.

To determine the effect of temperature on enzymatic activity, standard assay reaction mixtures were incubated at temperatures ranging from 20 to 45℃. In order to assess stability, rPDI was diluted to 1 μM while undiluted native PDI was used. The

rPDI and native PDI were incubated at temperatures from 10 to 70℃ for 3 hr and 1 hr respectively. The residual activity was measured following the procedure described above.

Results and discussion

1. Cloning of full-length PDI cDNA

The cDNA fragments were amplified by RT-PCR using RNA isolated from wheat roots. To isolate a full-length cDNA, we screened 3 wheat cDNA libraries using 1.5 kb cDNA fragments. The isolated cDNAs containing the PDI genes were sequenced. Three distinct sequences were identified; 2 of them (*wPDI 1* and *wPDI 3*) were 1548 bp in length, and the other (*wPDI 2*) was 1539 bp in length. The 3 sequences, *wPDI 1*, *wPDI 2*, and *wPDI 3* encode 515, 512, and 515 amino acid polypeptides, respectively. Each of these sequences had an encoded polypeptide with a putative signal sequence at the N-terminus and the ER retention signal KDEL at the C-terminus, as well as 2 thioredoxin-like catalytic sites (CGHC) and a putative N-glycosylation site (N-F-S) (Fig. 2). The wPDIs contained 5 domains: a, b, b′, a′, and c. The a and a′ domains had a thioredoxin-like catalytic site. The c domain was alanine rich, and alanine was 9 of 32, 7 of 29, and 9 of 32 amino acids in wPDI 1, wPDI 2, and wPDI 3, respectively. This feature appears to be conserved in wheat and barley. These results were nearly identical to those of M. CIAFFI[2]. The shared identity between wPDI 1 and wPDI 2 was 98.6% and that of wPDI 1 and wPDI 3 was 99.2%, while the shared identity between wPDI 2 and wPDI 3 was 99.0%. Ciaffi reported the sequences of 3 wheat protein disulfide isomerases (wheat PDI 1, 2, and 3), and wPDI 1 and wPDI 3 shared 99.6% identity with wheat PDI 3[2], while wPDI 2 shared 99.8% identity with wheat PDI 2[2]. However, we did not obtain any wPDI that was highly homologous to wheat PDI 1. The reason for this was that the 1 cultivar had numerous isotypes, at least 3. In the future we will try again to obtain the cDNA of wheat PDI 1.

Using phylogenic analysis based on deduced PDI

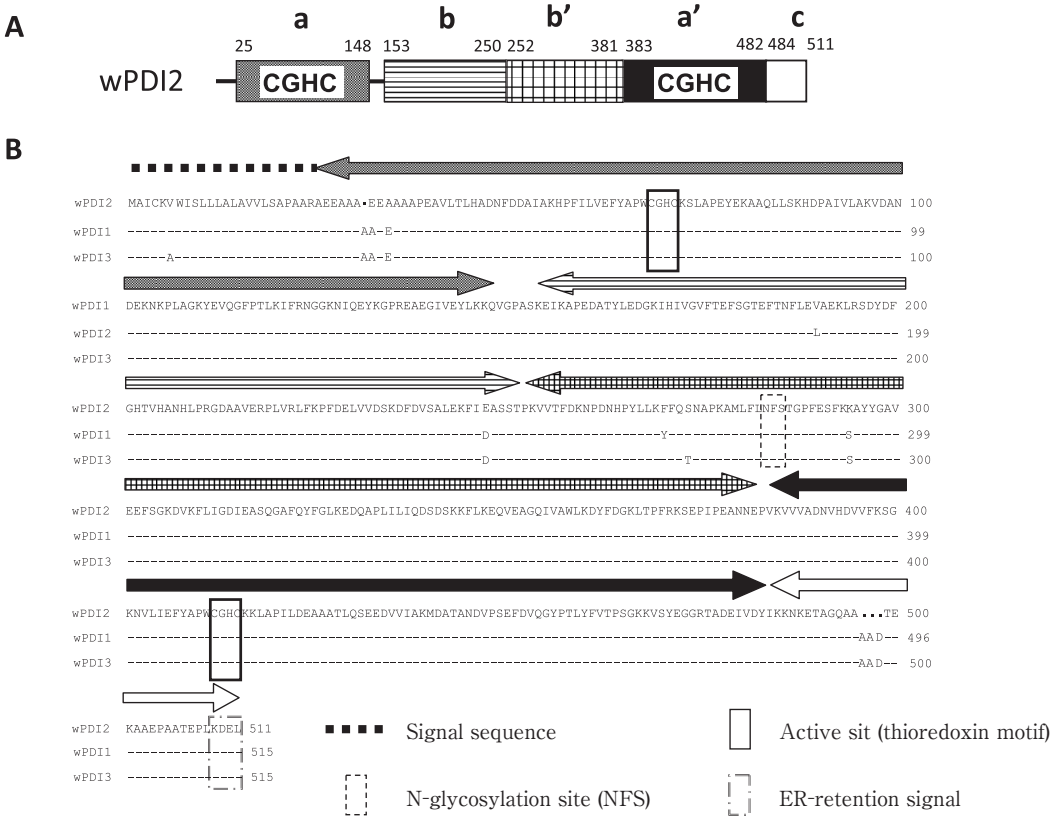

Fig. 2 Primary structure of wPDI

Domain organization of wPDI2 (A) and alignment of the 3 wPDI sequences (B). (A) The CGHC motifs (thioredoxin motifs) indicate the location of the active sites. (B) Active-site residues in the a and a' domains are shown as solid boxes. The dashed line box represents a putative N-glycosylation site (NFS). The double line box indicates the endoplasmic retention signal (KDEL).

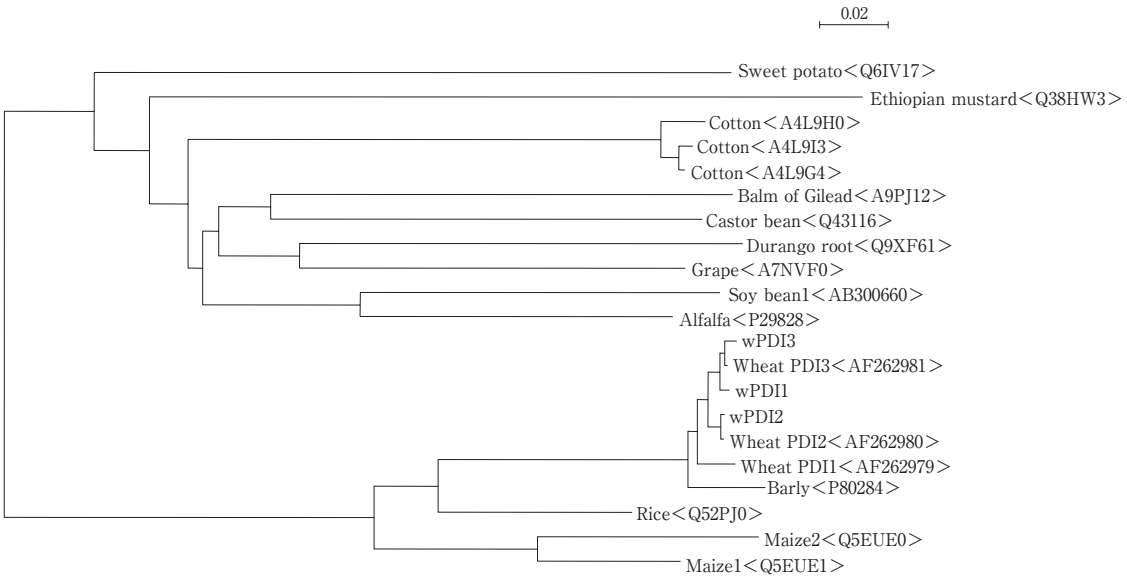

Fig. 3 The phylogenetic relationships of the amino acid sequences of the isolated wPDIs and some plant PDIs

The branching pattern was generated by the neighbor-joining method. The bar represents 0.02 nucleotide substitution per site. The sequence data were obtained from DDBJ.

amino acid sequences from wheat and related taxa, 2 potentially independent clusters were constructed (Fig. 3). One cluster contained the *Poaceae* family, including maize, and was located on an outer group in the cluster. All wheat PDIs were in a single subcluster in the family *Poaceae*, neighboring barley. Wheat PDI 1 reported by Ciaffi is somewhat far from the other wheat PDIs. However, the distances among PDI isotypes from maize and cotton were long compared with the distances among wheat PDIs. Therefore, this result may be attributed to species variety. The homology of PDI sequences of neighboring organisms in this group to wPDI was in the range from 96.3～96.7% (barley) to 79.8～81.0% (maize 2). In addition, the phylogenic tree based on PDI sequences had a shape similar to that of the taxonomic tree based on the DUF 642-domain gene family[30]. The 3-dimensional protein structure is shown in Fig. 4. The 2 thioredoxin-like catalytic sites face each other. Furthermore, analysis of the amino acid sequence of *Poaceae* PDI was carried out to investigate their 3-dimensional protein structure, and the results showed that the structure of *Poaceae* PDI was similar to that of the Poacese family (data not show). Hence, we propose that the PDI gene evolved naturally, similar to the above phylogenetic data[31].

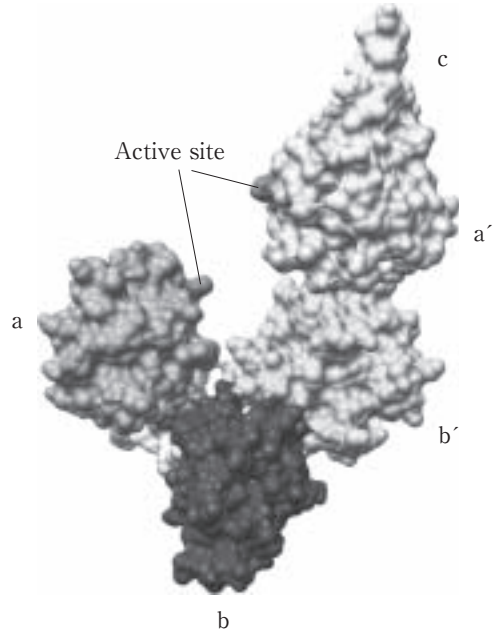

Fig. 4 Surface representation of PDI with the a, b, b´, and a´ domains in hot pink, blue, aquamarine, and pink, respectively, and the C-terminal extension in yellow

The side chains of the active site cysteines in the a and a´ domains are shown in red.

2. Expression and purification of Recombinant PDI

In this study, we obtained 3 PDI gene cDNAs from wheat. On the basis of phylogenic analyses, wPDI 2 was positioned at the center of the wheat PDI branch. In addition, this isotype did not diverge in cultivars, because the sequence was highly homologous to that of wheat PDI 2 reported by Ciaffi[2]; therefore, we chose to express wPDI 2.

Then, the presence and the location of the signal peptide cleavage sites of prePDI were determined using the Gram-negative prokaryote database in the SignalP 3.0 server[20]. The signal peptide of wheat PDI had a peptidase recognition site with high homology to the Gram-negative signal peptide (maximum cleavage site probability 99.9%). Therefore, we expressed rPDI with the wheat PDI signal peptide. The target protein was expressed in *E. coli* BL 21 (DE 3) and induced by IPTG. Next, rPDI was extracted from *E. coli*. SDS-PAGE showed that rPDI purified from the cell extracts using a TALON metal affinity resin column had a MW of approximately 63 kDa (Fig. 5) (Table 1). We confirmed removal of the rPDI signal peptide using N-terminal amino acid sequencing, which showed that rPDI did not have any signal peptide (data not show). The amount of purified recombinant PDI protein was 0.34 mg, and its total activity was 114.13 U. Further, 1 μM rPDI exhibited a specific activity of 332.74 U/mg. In addition, we tried to express PDI without a signal peptide under the same conditions. However, the resultant activity was 20% of the activity of PDI with the signal peptide (data not show). This may be due to the transport system of *E. coli*.

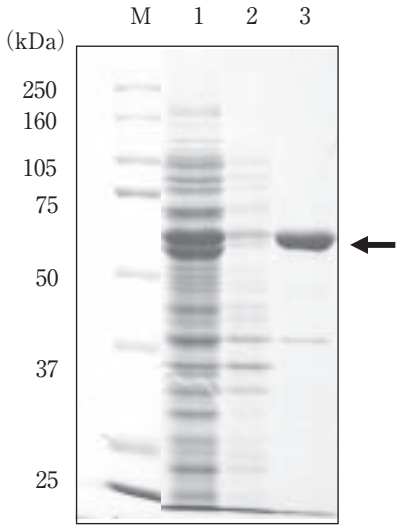

Fig. 5 Overexpression of rPDI from a pET21 a derivative in *E. coli*

SDS-PAGE analysis of the expressed and purified rPDI from *E. coli* harboring pET21 a/prePDI. Lane M, molecular weight markers (Precision Plus Protein Standards; Bio-Rad, USA); lane 1, cleared lysate; lane 2, inclusion bodies; lane 3, after TALON column chromatography.

Purified rPDI had an apparent MW of approximately 63 kDa as observed by SDS-PAGE, and a *p*I of 5.3 on a 2-dimensional gel, and the partially purified native PDI had a MW of approximately 63 kDa and a *p*I of 5.3 as observed by 2-dimensional gel electrophoresis and western blotting (data not show); thus, the molecular weight (MW) and *p*I values of rPDI and native PDI correspond to each other. Therefore, the carbohydrate moiety bound to N-F-S was found to be small, and it did not affect the MW or *p*I. Soybean, alfalfa, cattle, and yeast PDIs had MWs of 63 kDa, 60 kDa, 57 kDa, and 70 kDa, respectively[32)~35)] as determined by SDS-PAGE. Therefore, the molecular weight of wheat PDI was nearly identical to that of other plant PDIs. Meanwhile, the isoelectric point of wheat PDI was similar to those of cattle, yeast, and alfalfa, which were 4.2, 4.02, and 4.65, respectively[36),33),34)]. However, it differed from that of soybean PDI at 7.65[34)] (Table 2). The *p*I of wheat PDI was acidic, which is the case for most PDIs.

3. Analysis of folding activity of PDI (rPDI)

Protein disulfide isomerase (PDI) is an essential protein for the formation, reduction, or isomerization of protein disulfide bonds, thereby facilitating protein folding. This mechanism has been analyzed using several techniques. We employed a technique using disulfide reduced and denatured ovalbumin as a substrate, then CD spectrum was measured to detect conformational change.

We examined the capability of rPDI to catalyze the formation of disulfide bonds. In Fig. 6, protein folding for different times in the presence of rPDI is shown. At 5 hr, the refolded proteins from the rPDI reaction exhibited an intermediate CD spectrum at 212 nm, with about 50% of the

Table 1 Expression and purification of rPDI in *E.coli*

Purification step	Total protein (mg)	Total Activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract[a]	265.86	639.58	2.41	100
TALON metal affinity	0.34	114.13	335.67	17.85

[a] The starting material was crude extract from 100mℓ of induced *E. coli* culture.

Table 2 Characteristics of various PDIs

	molecular weight (kDa)	pI	Optimum pH	Optimum Temp. (℃)	Stability pH	Stability Temp. (℃)
Wheat (pr-PDI-His)	63 kDa	5.30	8.5	35	6～10	40
Wheat (native)	63 kDa	5.30	8.5	35	7～9	30
Soy bean	63 kDa	7.65	8.0	−	9～10	50
Yeast	70 kDa	4.02	8.5	−	−	＜55*
Cattle	57 kDa	4.20	7.5	−	−	−
Alfalfa	60 kDa	4.65	−	−	−	−
Chlamydomonas reinhardtii	60 kDa	−	7.6	30	−	−

*Yeast PDI was significantly inactivated when incubatedat 55℃ for 15 min.

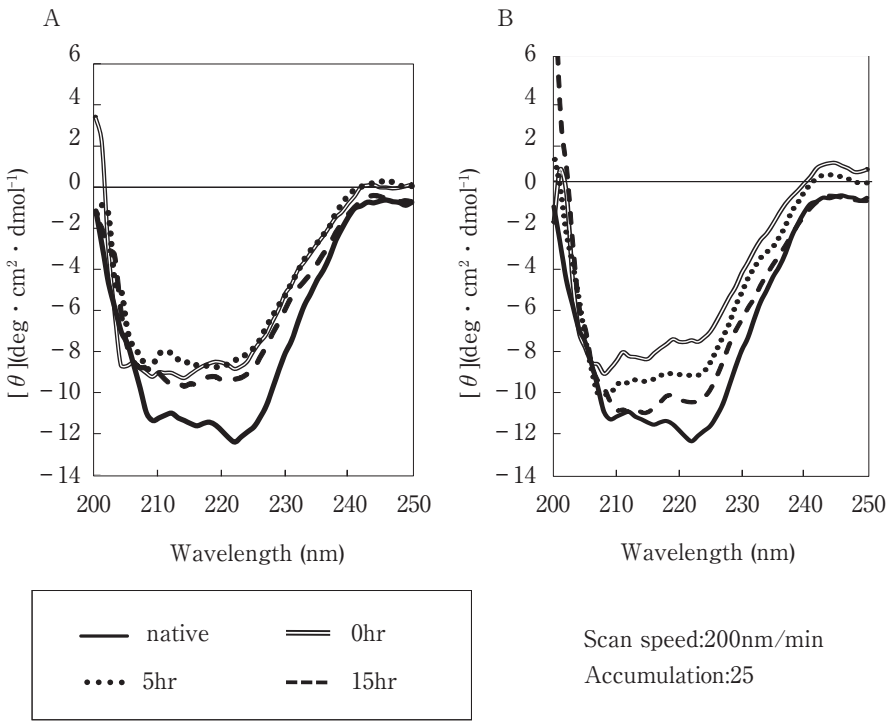

Fig. 6 The far UV CD spectra of ovalbumin in various states were recorded at 25℃ after 0, 5, and 15 hr

(A) in the absence of rPDI. (B) with the addition of rPDI (0.5 μM). (A) and (B) heavy line, native ovalbumin; dotted line, after 0 hr; double line, after 5 hr; solid line, after 15 hr

absolute ellipticity of the native form. The absolute ellipticity increased with increased folding time; and the CD spectra of proteins that were refolded for 15 hr were nearly identical to that of the native protein (Fig. 6 B). These results were similar to those of soybean PDI[32]. In contrast, reactions without prePDI did not show an intermediate CD spectrum, and did not change over time (Fig. 6 A). This shows that the rPDI catalyzed disulfide bond formation in the disulfide reduced and denatured ovalbumin. Ovalbumin was almost completely folded by rPDI in 15 hr, while soybean PDI took 20 hr[26]. However, the reaction conditions reported for soybean PDI were not clear.

4. Enzyme characteristics of Native PDI and rPDI

The activities of PDIs from various organisms (mammal, plant, fungi, yeast, and thermophiles) have been reported. However, few characteristics of PDIs have been reported (for example, soybean, and cattle), because the assay for PDI activity is complicated and the activity is generally very low. Therefore, data on PDI characteristics are very important for PDI research. In this study, we observed enzyme activity at various pHs and

temperatures.

The effect of temperature on PDI activity is shown in Fig. 7 a, and the results indicate that the optimal temperature for the 2 PDIs (rPDI and native) is 35℃. The 2 PDIs had similar activity versus temperature curves. The activity of the 2 PDIs was highest at pH 8.5. However, at alkaline pHs, the partially purified native PDI had higher activity than rPDI. In fact, at pH 10 the partially purified native PDI maintained 30% of the activity at optimal pH; however, no rPDI activity was detected at pH 10 (Fig. 7-b). The optimal temperature of the 2 wheat PDIs (35℃) was very similar to that of *Chlamydomonas reinhardtii* (30℃)[37]. The optimal pH of the 2 wheat PDIs at pH 8.5, was essentially the same as that of cattle PDI (pH 7.5)[34], Soybean PDI (pH 8.0)[32] and yeast PDI (pH 8.5)[32] (Table. 2). Regarding the enzyme stability at various temperatures and pHs, the enzyme retained more than 80% activity over the following conditions. The thermostability of rPDI was maintained up to 40℃, while the partially purified native PDI was maintained up to 30℃ (Fig. 7-c). At 50℃, the activity of rPDI and partially purified native PDI was 50% and less than 10% of that at the optimum temperature, respectively. Enzyme stability of rPDI was maintained between pH 6 and pH 9; while, the stability of partially purified native PDI was maintained between pH 7 and pH 9 (Fig. 7-d). Therefore, rPDI was superior to native PDI with respect to heat and pH stability. KISHI and WATAZU[38] reported that recombinant cholesterol dehydrogenase (CDH) was superior to native CDH, and they supposed that the reason for this was a difference in primary protein structure; hence, r-CDH had a higher order protein structure. Therefore, rPDI is thought to be superior to native PDI because of a difference in structure or the presence of the histidine tag. The pH-stability profile of wheat PDI was broad compared with the soybean PDI. In addition, wheat PDI was stable between neutral and alkaline pHs; while, soybean

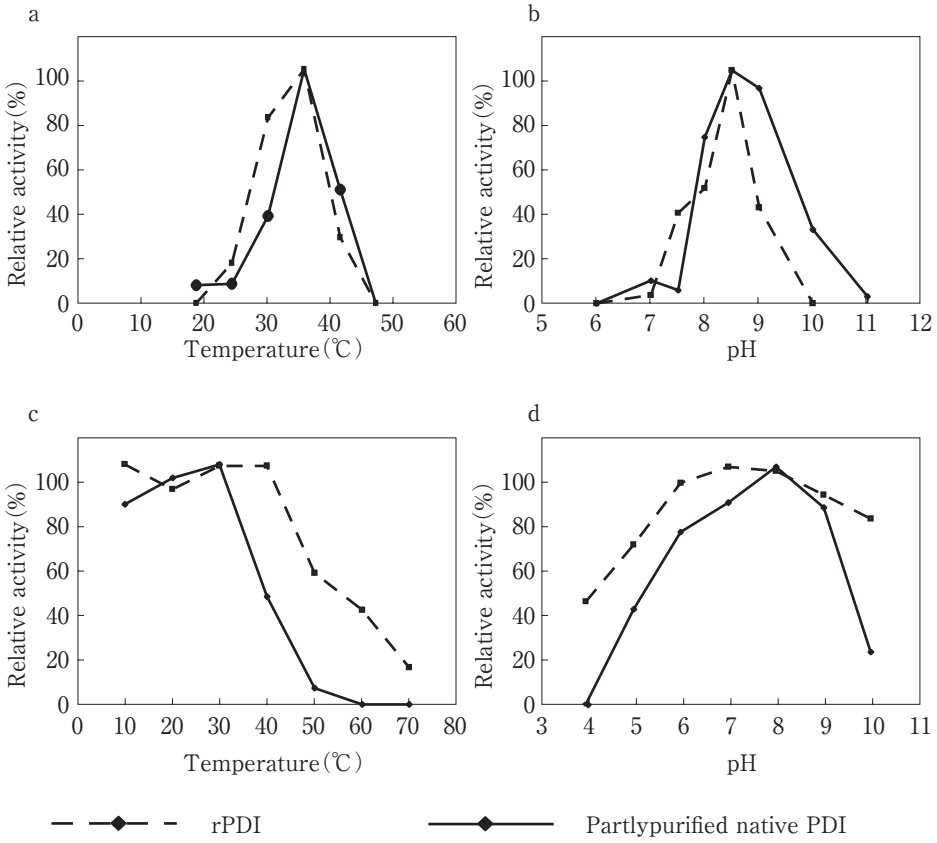

Fig. 7 Enzyme characterization of native PDI and rPDI

(A) Temperature dependency of rPDI and partially purified native PDI.
(B) pH dependency of rPDI and partially purified native PDI.
(C) Effect of temperature on the stability of rPDI and partially purified native PDI.
(D) Effect of pH on the stability of rPDI and partially purified native PDI.

PDI was stable only at alkaline pHs (9～10) (Table 2)[34]. Further, the native PDI was stable at 30℃ during a 1 hr incubation, while rPDI was stable at 40℃ during a 3 hr incubation. Heat treatment at 55℃ for 15 min significantly inactivated yeast PDI; however, the PDIs of cattle[34], thermophilic fungus[39], soybean[32], and wheat retained activity. In particular, rPDI was shown to be more stable than yeast PDI at high temperature, although it was less stable than soybean PDI at 50℃[32].

Conclusions

We obtained 3 distinct PDIs(wPDI 1, wPDI 2, and wPDI 3), and their amino acid sequences were similar to that of the previously reported wheat PDI[2]. wPDI 2 was positioned in the center of the wheat PDI branch in the phylogenic tree based on the amino acid sequences. In addition, this isotype did not diverge among cultivars, because it was highly homologous to the wheat PDI 2 reported by Ciffi[2]. Therefore, we chose to express wPDI 2 as rPDI with a signal peptide. The rPDI catalyzed the formation of disulfide bonds in disulfide reduced and denatured ovalbumin. The rPDI was superior to native PDI with respect to heat and pH stability, although the rPDI and native PDI were similar under optimum conditions. Recently, strong PDI activity has been reported in hard flour, semi-hard flour, and soft flour[29], and PDI is the only redox enzyme that is positively correlated with the ascorbic acid improver effect on bread quality[40]. Therefore, rPDI can be regarded as a bread improver. Future studies should be aimed at investigating the role of PDI in baking quality and the improver effect of rPDI.

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コムギ由来プロテインジスルフィドイソメラーゼの発現，精製と性状

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発芽・発根させたコムギ種子（品種ハルユタカ）の根部位よりRNAを抽出し，プロテインジスルフィドイソメラーゼ遺伝子（*wPDI1*～*3*）を取得した。*wPDI1*と*wPDI3*は1548bp，*wPDI2*は1539bpであり，アミノ酸レベルでの相同性は，wPDI1とwPDI2間で98.6%，wPDI1とwPDI3は99.2%，wPDI2とwPDI3は99.0%であった。*wPDI2*を用いて発現用ベクター構築した後，大腸菌を形質転換させリコンビナントPDI（rPDI）を調製した。インスリンを基質としたrPDIの総活性は114 U，比活性は336 U/mgであった。また，rPDIの酸化活性は，還元変性オボアルブミンのリフォールディングにて測定した。rPDIとコムギ粉中のPDI（native PDI）の性状を比較したところ，両PDIともに温度30℃，pH 8.5で最大活性を示したことから，rPDIはwPDIと類似の挙動を示すことを確認した。さらに，rPDIはnative PDIよりも温度，pHともに高い安定性を示した。本研究は，小麦由来PDIの発現に成功した初めての報告である。

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