Tissue and Spatial Distribution of Flavonol and Peroxidase in Onion Bulbs and Stability of Flavonol Glucosides during Boiling of the Scales

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Two major flavonol glycosides [quercetin 3,4′-diglucoside (F1) and quercetin 4′-monoglucoside (F2)] and a flavonol aglycon quercetin were mainly localized in the abaxial epidermis of scales. Their contents increased on aging. Peroxidase in scales oxidized flavonols in the order quercetin ≫ F2 > isoquercetin ≫ F1, and the activity was higher in the outer than in the inner scales. These results suggest that the enzyme can participate in the formation of defense substances against infection and brown compounds in the dry skin from quercetin. Contents of F1 and F2 in scales were decreased by cooking by boiling. This decrease was due to the release of F1 and F2 into cooking water and their oxidation. F2 was oxidized more rapidly than F1 during cooking. The difference in the stability between F1 and F2 was due to the presence or absence of a hydroxyl group at the C-3 position of the glucosides.

Keywords: *Flavonols; peroxidase; distribution in tissues; thermostability; onion (Allium cepa)*

INTRODUCTION

Flavonols are a group of phenolic compounds that are normally present in vascular plants. Their pharmacological functions have originally been discussed in relation to the decrease in the increased permeability and fragility of capillary to blood cells (Wagner, 1979). Their pharmacological functions have also been discussed in relation to the prevention of cataracts (Varma et al., 1975), hyperuricemic state (Bindoli et al., 1985), carcinogenesis (Fujiki et al., 1986), and cancer cell proliferation (Ramanathan et al., 1993). Recently, the above functions of flavonols have been reviewed by Caroll et al. (1997) and Hertog and Katan (1997). Furthermore, it has been found that some flavonols have antioxidative function. The function can be attributed to reduction of the superoxide anion radical (Sichel et al., 1991; Takahama, 1983) and radicals generated during lipid peroxidation (Jan et al., 1991; Pratt, 1965; Takahama, 1985; Younes and Siegers, 1981). Radicals of flavonols, which are formed during the antioxidative function, have been detected as UV−vis-transient spectra (Jan et al., 1991; Bors et al., 1997; Jovanovic et al., 1997) and EPR spectra (Bors et al., 1997). The antioxidative function of flavonol is enhanced by ascorbic acid (Jan et al., 1991; Sorata et al., 1988), and the enhancement is due to ascorbic acid-dependent reduction of phenoxyl radicals of flavonols (Jan et al., 1991; Sorata et al., 1988; Takahama and Oniki, 1997).

Because, as describe above, flavonols can function as antioxidants in vitro, we postulate that flavonols also have antioxidative function when absorbed into the human body. Flavonols may be absorbed from the intestine (Drieu et al., 1985). There are some reports about changes in blood levels of flavonols taken into a human body (Driue et al., 1985; Paganga and Rice-Evans, 1997) and their metabolism in mammals (Griffiths, 1982; Hollman and Katan, 1997). The presence of flavonols even in the bovine retina has been reported (Pautler et al., 1986).

The amount of quercetin glucosides in onion bulbs is much larger than that in other vegetables (Hertog et al., 1992; Mizuno et al., 1992), and the major glucosides are quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside (Price et al., 1997; Tsushida and Suzuki, 1995). The outermost scales of onion bulbs change to brown dry skin during aging or storage, and the glucosides of quercetin are transformed to quercetin during the dry skin formation (Bilyk et al., 1984; Patil and Pike, 1995). The transformation suggests that quercetin is included in the formation of brown compounds in the dry skin. As far as we know, it has not yet been published whether flavonols in onion bulbs are oxidized or not by peroxidase in onion bulbs. On the other hand, although onion is an important source of flavonols, there is only one report about changes in the contents of the flavonol glycosides during cooking (Price et al., 1997); these authors have reported no significant changes in the amounts of quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside during cooking by boiling.

One of the aims of the present study is the elucidation of the spatial and tissue distribution of flavonols and peroxidase in onion bulbs and the elucidation of substrate specificity of the peroxidase. The other aim is to determine whether contents of flavonols in scales are changed or not by cooking by boiling. On the basis of the results, we discuss the function of a flavonol/peroxidase system in onion and the usefulness of onion as a source of flavonols.

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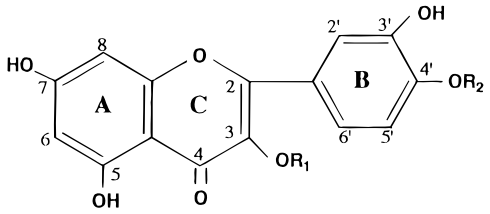

Figure 1. Structures of flavonols investigated in this study: F1, R_1 and R_2 = glucose; F2, R_1 = H and R_2 = glucose; isoquercitrin, R_1 = glucose and R_2 = H; quercetin, R_1 and R_2 = H.

MATERIALS AND METHODS

Plant Material and Part of Bulbs Used. Bulbs of onion (*Allium cepa* L. cv. Takanishiki) were obtained from a local market. A scale of the bulb is constituted from adaxial epidermis, abaxial epidermis, and mesophyll, and the contents of flavonols and the activity of peroxidase in these tissues were measured using first, third, and fifth scales from the exterior of a bulb. In addition, contents of flavonols and activity of peroxidase of the outermost scale of a bulb were also studied after the scale had been divided into four parts from the top to the base.

Preparation of Cell-Free Extracts and Measurements of Peroxidase Activity. Each material (0.1 g of fresh weight) described above was homogenized by a pestle and mortar in 3 mL of 50 mM sodium phosphate (pH 7.0) that contained 50 mg of PVP (insoluble). The homogenates were centrifuged at 10000g for 10 min at 4 °C. The supernatants were used as cell-free extracts. Peroxidase activity of the cell-free extracts was measured in the presence of 0.05 mM flavonol, 1 mM H_2O_2, and 0.1 mL of a cell-free extract in 50 mM sodium phosphate (pH 7.0) (Takahama and Egashira, 1991). Reactions were started by adding H_2O_2. Peroxidase activity was estimated from the initial absorbance decrease at peak wavelengths of flavonols. The wavelengths were 370, 352, 346, and 362 nm for quercetin, isoquercetin, F1, and F2, respectively. The molar absorption coefficient used was 8 mM^{-1} cm^{-1} for quercetin. The absorption coefficients for F1 and F2 used were 6 mM^{-1} cm^{-1}, which was estimated using isoquercetin as the standard.

Extraction of Flavonols. Flavonols of whole scales were extracted with 80% methanol. After 1 g of fresh weight of scales was homogenized in 80% methanol (8 mL) with a pestle and mortar on ice, the homogenate was centrifuged at 4500*g* for 1 min at 4 °C. The supernatant was dried with a rotary evaporator and dissolved in 0.5 mL of methanol. This solution was used to prepare F1 and F2 by TLC. Flavonols in each tissue and each position of scales were also extracted with 80% methanol as described above, and the extracts were used to quantify flavonols by HPLC (see below). Amounts of the extracts applied to an HPLC column were between 0.01 and 0.1 mL depending on flavonol concentrations of the extracts.

TLC. TLC was performed using ready-made TLC plates (silica gel 60, 0.5 mm thick; Merck, Darmstadt, Germany) using a mixture of water, methanol, and ethyl acetate (3:4:21, v/v) as a solvent. Two yellow bands that were flavonols [F1 (R_f = 0.2) and F2 (R_f = 0.6)] were extracted from the TLC plates with methanol and then concentrated under a stream of N_2 gas.

Identification of F1 and F2. Absorption spectra of F1 and F2 were measured with a double-beam spectrophotometer (557, Hitachi, Tokyo, Japan) in the presence of NaOH, sodium acetate, boric acid, or aluminum chloride (Markham, 1989). The results obtained suggest that both F1 and F2 have no *o*-dihydroxyl groups in the B-ring and that F2 has a hydroxyl group at C-3 and no free hydroxyl group at C-4′ (Figure 1). F1 and F2 were hydrolyzed in 1 M HCl (5 mL) in boiling water for 30 min and extracted with ethyl acetate. The water layer was used to identify sugars by a ready-made cellulose TLC plate (0.1 mm thick; Merck) using a mixture of *n*-butanol, acetic acid, and water (9:5:8, v/v) as a solvent. Sugars were visualized by spraying a mixture of phthalic acid (1.7 g) and aniline (0.93 g) dissolved in 100 mL of *n*-butanol saturated with water. Only glucose was detected. Aglycons in the ethyl acetate layer were identified by an HPLC system [Shim-pack CLC-ODS (150 × 6 mm i.d.) combined with spectrophotometric detector with a photodiode array (SPD-M1A); Shimadzu, Kyoto, Japan]. A mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (3:2, v/v), and the flow rate was 1 mL min^{-1}. Only quercetin was detected. F1 and F2 were also hydrolyzed under mild conditions (at 60 °C in 1 M HCl). After defined periods of incubation, portions of the solution (0.02 mL) were withdrawn and analyzed by HPLC using a mixture of methanol and 25 mM KH_2PO_4 (1:1, v/v) as a mobile phase. Products formed during the hydrolysis of F1 were isoquercitrin, F2, and quercetin, and the product formed during the hydrolysis of F2 was only quercetin. F1 and F2 were hydrolyzed to quercetin by *β*-glucosidase, indicating that both F1 and F2 were quercetin glucosides. From the above results, we tentatively identified F1 as quercetin 3,4′-diglucoside and F2 as quercetin 4′-monoglucoside. It has been reported that onion bulbs contain quercetin 3,4′-diglucoside and quercetin 4′-monoglucoside as major flavonols (Price et al., 1997; Tsushida and Suzuki, 1995).

Quantification of F1 and F2 by HPLC. Flavonols extracted from various tissues and positions of scales were separated by HPLC using a mixture of methanol and 25 mM KH_2PO_4 (1:1, v/v) as a mobile phase. The methanol extracts contained two major flavonols of which retention times and absorption spectra were identical with those of F1 and F2. Their contents in each material were then estimated from areas under peaks on chromatograms at 360 nm using isoquercitrin as the standard.

Thermostability of F1 and F2. There is a question whether contents of F1 and F2 in scales change or not during cooking by boiling. To examine this, the first scale of a bulb (∼7 cm in height and ∼22 cm in circumference at the equator) was longitudinally divided into eight. Six pieces (∼20 g of fresh weight) were boiled in 200 mL of water, and the remaining was used to determine the initial contents of F1 and F2. After boiling for defined periods, flavonols in scales were extracted with 80% methanol and their contents were determined by HPLC as described above. Contents of F1 and F2 in cooking water were also estimated by HPLC.

Thermostability of extracted F1 and F2 was also studied. F1 and F2 were dissolved in distilled water and faucet water at a concentration of 0.1 mM, and the solutions were heated for 1 h in a boiling water bath. F1 and F2 were quantified by HPLC as described above.

Estimation of Concentrations of Fe^{3+}. Concentrations of Fe^{3+} in faucet water were estimated by measuring the absorbance at 470 nm due to formation of a complex between Fe^{3+} and SCN^-. The mixture (3 mL) contained 1.5 mL of 6% HNO_3, 1.2 mL of mineral or faucet water, and 0.3 mL of 1 M NaSCN. A calibration curve was determined using $FeCl_3$.

Reagents. Quercetin was obtained from Wako Pure Chemical Industries (Osaka, Japan), and isoquercitrin (quercetin 3-glucoside) was from Extrasyntése (Genay, France). *β*-Glucosidase was from Oriental Yeast Co. (Osaka, Japan). Mineral water was from House Shokuhin Co. (Osaka, Japan).

RESULTS

Distribution of F1, F2, and Peroxidase in Scales. The contents of F1 and F2 were highest in abaxial epidermis and lowest in mesophyll in any scales, and the contents in each tissue decreased from the outer to the inner scales (Table 1). The decrease from the outer to the inner scales has been reported by Bilyk et al. (1984), Patil and Pike (1995), and Starke and Herrmann (1976). When the contents of F1 and F2 were measured from the top to the base of scales, their contents were highest at the top position and their contents in other positions were similar to one another (Figure 2). The molar ratios of F1/F2 were decreased from the inner

Table 1. Relative Levels of Flavonols in Epidermis and Mesophyll[a]

flavonol	scale[b]	abaxial epidermis	adaxial epidermis	mesophyll
F1	first	100	2.15 ± 1.95	0.24 ± 0.18
	third	74 ± 26	0.19 ± 0.01	0.04 ± 0.01
	fifth	47 ± 31	0.28 ± 0.30	0.03 ± 0.01
F2	first	100	1.60 ± 0.75	0.13 ± 0.02
	third	75 ± 20	0.17 ± 0.05	0.03 ± 0.01
	fifth	38 ± 25	0.20 ± 0.17	0.02 ± 0.01
quercetin	first	100	BD	BD
	fifth	47 ± 23	BD	BD

[a] Levels of F1, F2, and quercetin of abaxial epidermis of the first scales were 38 ± 13, 25 ± 8, and 0.42 ± 0.11 μmol (g of fresh weight)$^{-1}$. Values are means ± SDs (n = 3). BD, below detection. [b] From exterior to interior.

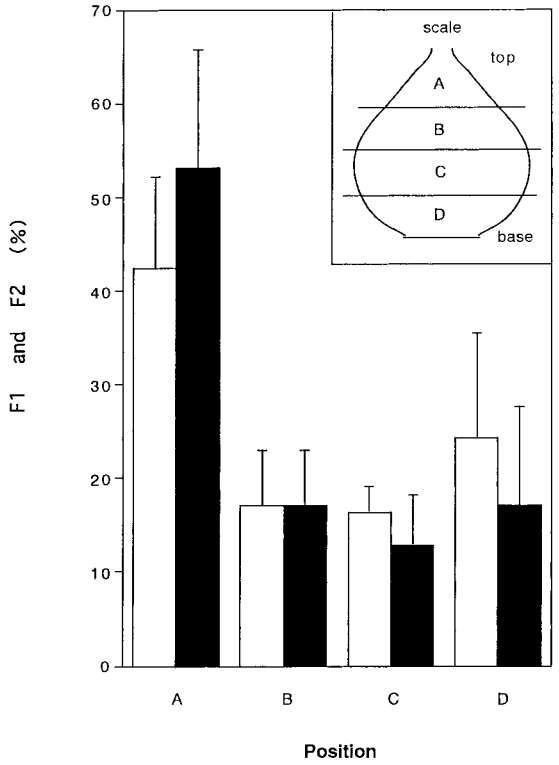

Figure 2. Changes in levels of F1 and F2 from the top to the base of scales. The first scale was divided into four parts from the top (A) to the base (D) as shown in the inset. White column, F1; black column, F2. Values of F1 and F2 at each part are expressed relative to sum of each flavonol of the four parts. The total levels of F1 and F2 were 0.65 ± 0.31 and 0.48 ± 0.26 μmol (g of fresh weight)$^{-1}$ (n = 3 or 4), respectively.

(fifth scale, 2.9 ± 1.0) to the outer (first scale, 1.5 ± 0.3) scale and increased from the top (position A in Figure 2, 1.1 ± 0.1) to the base (position D in Figure 2, 2.3 ± 0.7). Quercetin was found in the abaxial epidermis, although the contents were much less than those of F1 and F2 as reported by Kiviranta et al. (1988), and the concentrations of quercetin in cells were calculated to range from 0.1 to 0.6 mM.

Peroxidase activity in different tissues of a scale was in the order adaxial epidermis > abaxial epidermis > mesophyll. Rates of oxidation of quercetin by the peroxidase were about 20- and 100-fold faster than those of oxidation of F2 and isoquercitrin, respectively, in every tissue extract (data not shown). No detectable oxidation of F1 by peroxidase from each tissue was observed. Peroxidase activity was much higher in the first scale than in the fifth scale (Table 2), and the

Table 2. Peroxidase Activity in Various Tissues

	μmol of quercetin oxidized (g of fresh weight)$^{-1}$ min^{-1}	
	first scale	fifth scale
abaxial epidermis	3.96 ± 0.76	0.18 ± 0.03
adaxial epidermis	6.97 ± 0.30	0.91 ± 0.24
mesophyll	2.94 ± 0.85	0.18 ± 0.06

[a] Values were means ± SD (n = 3).

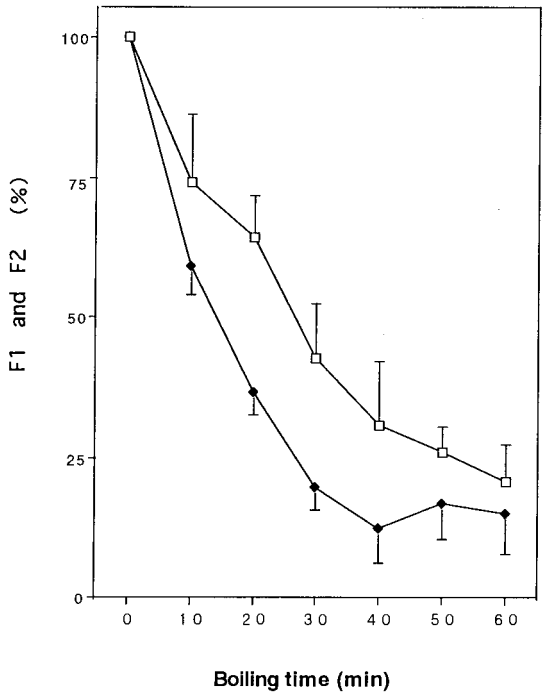

Figure 3. Changes in amounts of F1 and F2 in scales as a function of boiling time: open symbols, F1; solid symbols, F2; bars, SDs (n = 3 or 4). The initial levels (before boiling) of F1 and F2 were 0.12 ± 0.02 and 0.11 ± 0.02 μmol (g of fresh weight)$^{-1}$, respectively.

activity at the top of the scale (position A in Figure 2) was ~1.2-fold higher than that at the base of the same scale (position D in Figure 2).

Changes in F1 and F2 by Cooking. Contents of F1 and F2 in scales decreased during cooking by boiling in distilled water. Cooking periods required for the decrease to half of the initial contents were about 25 and 15 min for F1 and F2, respectively (Figure 3). We examined whether the decrease in the contents of F1 and F2 was due to the degradation or the release from the scales to cooking water. The result in Figure 4 indicates that both F1 and F2 were found in cooking water as well as in scales. The total amount of F1 (amount in boiled scales plus amount in cooking water) after a 1 h treatment was nearly the same as the initial amount in the scales before cooking. The total amount of F2 decreased somewhat by cooking for 1 h. It has been reported by Price et al. (1997) that the proportion of F1 and F2 leached into the cooking water was unchanged from that in the onion scale.

Amounts of F1 and F2 in scales and in water were also measured after scales had been boiled in mineral water and faucet water for 1 h (Figure 4). Here again, F1 and F2 were leached from scales into cooking water. The total amount of F1 was not significantly changed and decreased to ~80% of the initial amount when boiled in mineral and faucet water, respectively. The

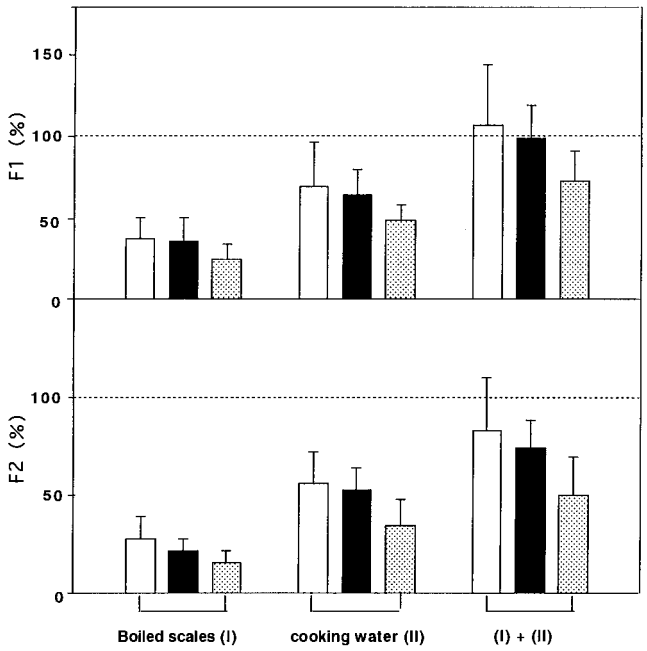

Figure 4. Amounts of F1 and F2 in scales and cooking water after boiling of scales. Values are expressed relative to the levels of flavonols in scales before boiling. Scales were boiled for 1 h in distilled water (white column), mineral water (black column) and faucet water (dotted column). Initial levels (before boiling) of F1 (upper panel) were 0.31± 0.12, 0.25 ± 0.09 and 0.30 ± 0.06 and those of F2 (lower panel) were 0.22 ± 0.04, 0.21 ± 0.07 and 0.24 ± 0.03 μmol (g of fresh weight)$^{-1}$ for white, black, and dotted columns, respectively. Bars, SDs (n = 3).

total amount of F2 was decreased to about 80 and 50% of the initial amount by boiling in mineral water and faucet water, respectively. The pH values of the water in which scales were boiled were 5.6, 7.2, and 6.3 for distilled water, mineral water, and faucet water, respectively. This result indicates that the difference in the stability is not due to the difference in the pH of the cooking water.

When F1 that was dissolved in distilled water and faucet water was heated for 1 h in a boiling water bath, F1 was practically not degraded. However, the concentration decreased to ~85% and <10% of the initial concentration when F2 that was dissolved in distilled water and faucet water, respectively, was heated for 1 h. It is known that faucet water contains Fe^{3+} released from steel pipe and chlorine added for sterilization. The concentration of Fe^{3+} in the water ranged from 0.5 to 1 μM and that of chlorine, which was in equilibrium with HOCl, was ~7 μM according to the data from the city of Kitakyushu (Japan). We then examined the effects of the above oxidants on the degradation of F1 and F2 during boiling in distilled water. The oxidation of F1 was affected neither by 1 μM $FeCl_3$ nor by 10 μM NaOCl, and the oxidation of F2 was facilitated ~10% by 10 μM NaOCl but not by 1 μM $FeCl_3$.

DISCUSSION

It has been discussed that flavonols can protect plant cells from UV light. This discussion is based on the observation that flavonols, which absorb UV-BB light, are present in the epidermis of leaves (Dixon and Paiva, 1995; Hrazdina, 1992). The localization agrees with our observation in this study. In addition, flavonols can function to scavenge hydrogen peroxide formed in plant cells cooperating with peroxidase and ascorbic acid (Takahama and Oniki, 1997). As long as ascorbic acid is present, the accumulation of oxidation products of flavonols is very slow, and oxidation products of flavonols start to be accumulated when concentrations of ascorbic acid are decreased to nearly zero.

Concentrations of F1, F2, and quercetin were higher in the outer than the inner scales (Table 1) and at the upper than the lower positions of a scale (Figure 2). These results suggest that contents of flavonols increase during aging of cells that constitute scales of onion bulbs because it is known that cells of the outer scales are more aged than those of the inner scales in a bulb and that cells of the upper position are more aged than those of the lower position in a scale (Barden et al., 1987). In addition, molar ratios of F1/F2 decreased as a function of age. This result suggests that, during aging of scales, F2 is synthesized more rapidly than F1 and/or that glucose at the C-3 position of F1 is enzymatically hydrolyzed, producing F2 as discussed by Tsushida and Suzuki (1996). F2 may be transformed to quercetin by enzymatic hydrolysis (Tsushida and Suzuki, 1996). As a result, quercetin may be accumulated in the dry skin of onion bulbs during aging as reported by Bilyk et al. (1984) and Tsushida and Suzuki (1996).

Peroxidase of onion scales rapidly oxidized quercetin but not the glucosides, and high activity of the enzyme was found in the outer scales (Table 2). From these data and the data that more quercetin was present in the outer than in the inner scales (Table 1), we suppose that onion peroxidase can oxidize quercetin in cells if hydrogen peroxide is supplied. It has been reported that formation of hydrogen peroxide is stimulated on injury of plant cells (Mehdy et al., 1996; Lamb and Dixon, 1997). Such oxidation reaction leads to the formation of polymers and degradation products from quercetin (Schreier and Miller, 1985) that may relate to wound healing or protection of the plant from infection. Because, under normal conditions, ascorbic acid is present in plant cells and oxidized forms of ascorbic acid, monodehydroascorbic acid and dehydroascorbic acid, are enzymatically reduced to ascorbic acid (Halliwell and Gutteridge, 1989), the peroxidase/quercetin/ascorbic acid system can function to scavenge hydrogen peroxide that is not used for physiological reactions. Physiological reactions such as lignin formation and cross-link formation require hydrogen peroxide (Sánchez et al., 1997; Takahama, 1993). Alternatively, the system may participate in the regulation of cellular levels of hydrogen peroxide. The peroxidase-dependent oxidation of quercetin can also contribute to the formation of brown compounds in the dry skin during aging, because levels of hydrogen peroxide are increased (Schopfer et al., 1994) and those of ascorbic acid of onion scales (unpublished result) were decreased during aging. In addition to quercetin, at present, we cannot exclude F2 as a substrate for peroxidase in scales because its level was ~50 times higher than quercetin (Table 1).

The data in Figures 3 and 4 indicate that F1 is more stable than F2. The difference in the stability is attributed to the presence and absence of the hydroxyl group at the C-3 position. The faster oxidation of F2 than F1 catalyzed by onion peroxidase indicates that the hydroxyl group of the C-3 position is attacked by the enzyme. It has been reported that the hydroxyl group of flavonols is oxidized by active oxygen species

such as O_2^- (Nishinaga et al., 1979; Takahama, 1987) and singlet molecular oxygen (Matuura et al., 1970), dissociating flavonols into compounds derived from A- and B-rings.

When scales were boiled in faucet water, F2 degraded rapidly, and F2 dissolved in faucet water was also degraded rapidly by heating. Because heat-dependent degradation of F2 was not significantly affected by 1 μM $FeCl_3$ and 10 μM NaOCl, neither chlorine nor Fe^{3+} is important to enhance the degradation of F2. At present, we have no idea what components in faucet water cause the enhancement of the degradation. The decrease in F2 when scales were boiled for 1 h in faucet water (~50%) was smaller than that in F2 when F2 dissolved in faucet water was boiled for the same period (~90%). The difference in the stability between the two treatments may be due to the presence or absence of reductants that can reduce the radical of F2 generated during heating. Ascorbic acid is one of the candidates, because the acid can effectively reduce phenoxyl radicals (Takahama and Oniki, 1997; Yamasaki et al., 1998) and *o*-quinones (Takahama, 1986). The content of the acid in scales ranged from 0.1 to 0.4 nmol (g of fresh weight)$^{-1}$.

As discussed above, onion bulbs contain a thermostable quercetin glucoside (F1) at high concentrations. Therefore, we can suppose that the glucoside will not be oxidized so much during cooking by boiling. The thermostability was due to the absence of the hydroxyl group at the C-3 position. In this way, onion bulbs are an important source of flavonols even after cooking by boiling if we take not only scales but also the broth.

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