accelerated storage conditions used in this study. Therefore, any increase in these late-eluting peaks must be due to highly nonpolar browning pigments. These peaks appeared to be quite large in glass-stored juice in comparison to juice stored in tin-plated cans. (The trend for the nonpolar pigments in glass-stored juice could not be accurately quantified and, therefore, is not shown in Figure 5.) The trend for nonpolar pigment peaks from cans is shown in Figure 5. There is a dramatic increase in these pigments with increasing storage time. These late-eluting, nonpolar pigments appear to be a major contributor to the browning observed in canned grapefruit juice. It can be seen from Figures 2 and 3 that the late-eluting, nonpolar pigments in glass-stored grapefruit juice also constitute an important component of the total browning observed.

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Effect of γ-Irradiation on Thiol Compounds in Grapefruit

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The effect of ^{60}Co γ-irradiation on thiol compounds in grapefruit was investigated. Thiols were separated by HPLC and measured with a fluorescence detector. Reduced glutathione (GSH), cysteine (CySH), cysteinylglycine (CySGly), and a number of unknown peaks were observed in unirradiated grapefruit. GSH was the main thiol at an average concentration of 143.3 μM. GSH content exponentially decreased with increased radiation doses, and after 100 krad only 80% of the original remained. The *G* value based on the result of 100 krad was 0.29. Authentic GSH in water or citrate buffer (pH 3) was converted mainly to its oxidized form (GSSG) with γ-irradiation. GSSG in irradiated grapefruits showed no equivalent increase, however.

Recent animal feeding studies have explicitly proved the safety of irradiated foodstuffs (International Food Irradiation Project, Report R51, 1979). On the basis of these results, all foods irradiated below 10 kGy (1 Mrad) were recognized as suitable for human consumption by a Joint Expert Committee of the International Atomic Energy Agency (IAEA), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO). This decision and progress in irradiation technique development soon led to the appearance of irradiated foodstuffs on the international market. An identification method for irradiated foods thus became desirable to distinguish them from unirradiated foods.

Various components (proteins, starch, vitamins, carbohydrates, lipids, etc.) (Elias and Cohen, 1983; Stockhausen and Bögl, 1978; Simic, 1978; Schubert et al., 1973; Drijver et al., 1986; Moshonas and Shaw, 1982, 1984) were analyzed in food before and after irradiation. The use of measurements of such components for identification however was limited because changes could also occur in these compounds as a result of heating or storage conditions.

As a part of our research on the identification of irradiated foodstuffs, the differences between the components in grapefruit before and after irradiation have been investigated. Among various components in grapefruit, thiols seem to be one of the most sensitive group of compounds, as suggested by radiation biochemical studies (Meyers and Bide, 1966; Sutherland and Pihl, 1968). The determinations of thiol compounds are carried out generally by the following methods: electrochemical detection (Saetre and Rabenstein, 1978; Kreuzig and Frank, 1981; Bergstrom et al., 1981; Shimada et al., 1982), UV–vis labeling (Kuwata et al., 1982; Chang et al., 1983; Nishiyama and Kuninori, 1984; Moroney et al., 1984), and fluorescence labeling (Kanaoka, 1980; Lankmayr et al., 1981; Newton et al., 1981; Werkhoven-Goewie et al., 1981). The most appropriate method of estimating the individual thiol seems to be fluorometry due to its selectivity and sensitivity.

The derivatizing agent ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) (Imai et al., 1983; Toyo'oka and Imai, 1984) was used in this study. It is highly soluble in water, is selective with respect to thiol-containing compounds, and has well-defined fluorescence characteristics (excitation at 380 nm, emission at 510 nm) (Figure 1); its

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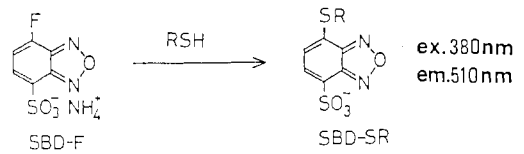

Figure 1. Structure of SBD-F and its reaction with thiols.

derivatives are stable for more than 1 week at 4 °C. The detection limits (signal to noise ratio of 2) of SBD-SR after separation by high-performance liquid chromatography (HPLC) were in the range 0.1–1 pmol (Toyo'oka and Imai, 1983; Toyo'oka et al., 1984). SBD-F was used as a precolumn derivatization reagent for HPLC determination of various thiols such as cysteine (CySH) and reduced glutathione (GSH) in human blood (Toyo'oka and Imai, 1983). These applications facilitated and determination of thiol compounds in grapefruit.

This paper describes the HPLC determination of thiols in grapefruit pulp and the effects of γ-irradiation on the thiols.

EXPERIMENTAL SECTION

Materials and Reagents. Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate and SBD-Cys were synthesized and purified according to the procedure described previously (Imai et al., 1983; Toyo'oka and Imai, 1984). SBD-F is now commerically available from Dojindo Laboratories (Kumamoto, Japan). Homocysteine (HCySH), cystine (CySSCy), coenzyme A (CoA), D-pantethine, and nucleotide pyrophosphatase (from Crotalus Adamanteus Venom, Type II) were purchased from Sigma Chemical Co (St. Louis, MO). 2-Mercaptoethanol (ME), cysteine hydrochloride (CySH·HCl), *N*-acetylcysteine (*N*-AcCySH), and glutathione (reduced (GSH) and oxidized (GSSG)) were from Wako Pure Chemicals (Osaka, Japan). Tri-*n*-butylphosphine (TBP) (Nakarai Chemicals, Kyoto, Japan), ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) (Kanto Chemicals, Tokyo, Japan), and cystinyldiglycine (Vega Biochemicals, Tucson, AZ) were used as received. Phosphoric acid (H_3PO_4) used was of biochemical reagent grade (Wako). Acetonitrile (CH_3CN) and water (H_2O) were HPLC grade (Wako). All other chemicals were of analytical reagent grade and were used without further purification. Tested grapefruits (Marsh white seedless) were harvested in California in 1986.

Apparatus. A Biotron homogenizer (Kussnacht, Switzerland) was used for the mixing of grapefruit pulp. A Hitachi 05P-21 centrifuge (Tokyo, Japan) and a Yamato MT-31 mixer (Tokyo, Japan) were also employed. Reaction temperature for the derivatization with SBD-F was controlled by a water bath, Model BM-41 (Yamato, Tokyo, Japan).

Irradiation. The γ-irradiation doses were between 30 and 1000 krad at room temperature. The doses were controlled by the change of both exposure time and distance from ^{60}Co γ-source. Irradiation facilities were provided by the Japan Atomic Energy Research Institute (Takasaki Radiation Chemistry Research Establishment).

HPLC. A Model 655A-12 high-performance liquid chromatograph (HPLC) (Hitachi, Tokyo, Japan) equipped with a Rheodyne injector (Model 7125; Cotati, CA) were used. The gradient elution was controlled by an L-5000 LC controller (Hitachi), and the data were calculated by a D-2000 chromatointegrator (Hitachi). An Inertsil ODS column (150 × 4.6 mm (i.d.), 5 μm) (Gasukuro Kogyo, Tokyo, Japan) was used. A Hitachi F-1000 fluorescence spectrophotometer equipped with a 12-μL flow cell was used for detection of the eluate with excitation at 380 ± 7.5 nm and emission at 510 ± 7.5 nm. In a separate experiment, the column eluate was monitored at λ 210 nm (655A UV monitor; Hitachi). Column temperature was fixed at 40 °C by a column oven (Gasukuro Kogyo). All the mobile phases were degassed with an on-line degasser (Shodex KT-31; Showa Denko, Tokyo, Japan) before the pump unit. The linear gradient elutions from (A) 0.15 M H_3PO_4–CH_3CN (97:3) to (B) 0.15 M H_3PO_4–CH_3CN (80:20) over 30 min and B to A over 5 min were adopted for the separation of the SBD-SR. The flow rate of the eluent was 1.0 mL/min.

Derivatization and HPLC Separation of Thiol Compounds. A 300-μL portion of SBD-F (12 mM) in 0.1 M borax (pH 9.3) containing 5 mM Na_2EDTA was added to 0.1 mL of the mixed solution of thiols (5 μM each CySH, HCySH, GSH, *N*-AcCySH, and ME) and reacted at 60 °C for 60 min. Of the mixed solution of disulfides (2.5 μM each cystinyldiglycine, pantethine, lipoic acid, and lipoamide), 0.1 mL was reacted with 0.28 mL of SBD-F (12 mM) in 0.1 M borax (pH 9.3, 5 mM Na_2EDTA) in the presence of 20 μL of 10% TBP in acetonitrile (CH_3CN). 4′-Phosphopantetheine (4′-PP) was prepared by hydrolysis (37 °C for 80 min) of 1 mg of CoA with 1 mg of nucleotide pyrophosphatase (7.2 units/mg of protein) in 1 mL of 0.1 M NH_4HCO_3 (pH 7.6) containing 0.1 M $MgCl_2$ (Wang et al., 1954). Four milliliter of CH_3CN was added to the hydrolysate, and the solution was centrifuged at 3000 rpm for 5 min. A 20-μL portion of the supernatant was reacted with 0.5 mL of SBD-F (5 mM) in 0.1 M borax (pH 9.3, 5 mM Na_2EDTA). The CoA blank without the enzyme was treated and derivatized in the same manner. An aliquot of each solution derivatized with SBD-F was applied to the HPLC column, and the retention times of SBD-SR were determined. The void volume of the column was measured with SBD-Cys as a marker under elution with 100% CH_3CN.

Determination of Thiols and Disulfides in Grapefruit Pulp. Portions of 10 mL of peeled grapefruit homogenated pulp were transferred to a glass tube containing 0.1 g Na_2EDTA. The tubes were placed in single layers and irradiated at dosages of 0, 50, 100, 200, 500, and 1000 krad (this sample designated as the "irradiated pulp"). All samples were irradiated in air without nitrogen flushing. Whole grapefruits (0.4–0.6-kg weight) were also irradiated at 0, 30, 50, 100, and 200 krad in the same manner (designated as "irradiated grapefruit"). At fixed time intervals (0, 15, 30, and 45 days), the grapefruits were peeled and the pulp was homogenized after the addition of 1 g of Na_2EDTA. The irradiated pulp and irradiated grapefruit samples were centrifuged at 3500 rpm for 10 min, and 0.1 mL of each supernatant was diluted to 1.0 mL with H_2O containing 5 mM Na_2EDTA. Subsequently 0.1 mL of the diluted solution was added to 0.1 mL of HCySH (3.9 μM, internal standard (IS)) and 0.2 mL of SBD-F (20 mM) in 0.1 M borax (pH 9.3) containing 5 mM Na_2 EDTA. Another 0.1 mL of diluted solution was added to 0.1 mL of HCySH (3.9 μM), 0.18 mL of SBD-F (20 mM) in 0.1 M borax (pH 9.3, 5 mM Na_2EDTA), and 20 μL of 10% TBP in CH_3CN. Each solution was heated at 60 °C for 60 min. After the reaction, 20 μL of cooled solution was injected onto the HPLC column.

Calibration Curve for Thiols and Disulfides. The 0.1-mL solutions spiked with known amounts of authentic thiols (2–100 μM CySH and GSH) or disulfides (1–50 μM CySSCy and GSSG) were analyzed according to the procedure for the determination of thiols in grapefruit. The ratios of peak areas of thiol or disulfide to internal standard (IS) were plotted against the amounts of the added CySH, GSH, CySSCy, or GSSG.

Effect of γ-Irradiation on Authentic GSH. GSH (1.1 mM) dissolved in H_2O or 0.1 M citrate buffer (pH 3.0) was γ-irradiated at 100 and 200 krad (designated as "authentic GSH"). The solutions were immediately analyzed with HPLC followed by UV detection at 210 nm. The amount of GSSG oxidized with the irradiation of GSH was calculated on the basis of the peak area of authentic GSSG.

HPLC conditions: column, Inertsil ODS (150 × 4.6 mm (i.d.), 5 μm) at 40 °C; eluent A, H_2O; eluent B, MeOH; gradient elution, from 100% A to 90% B over 60 min; flow rate, 1.0 mL/min.

RESULTS AND DISCUSSION

Identification of Thiol Compounds in Grapefruit Pulp. Our first effort was focused on the HPLC separation of some thiols derivatized with SBD-F. Since SBD-SR have a sulfonic acid group (SO_3^-) in the structure, the derivatives were eluted near the void volume of the column (Inertsil ODS) with neutral solutions (pH 4–7) and the appropriate separation was not obtained from the solutions. The acidic eluents gave the larger capacity factors (k') for all the derivatives than the neutral eluents. The complete separation was not obtained from the isocratic elutions using 0.15 M H_3PO_4 containing various concentrations of CH_3CN. Therefore, a linear gradient elution

Table I. Retention Times (t) and Capacity Factors (k') for Some SBD-SR Derivatives[a]

thiol	t, min	k'
cysteine (CySH)	4.7	1.35
cysteinylglycine (CySGly)	7.0	2.50
homocysteine (HCySH)	8.9	3.45
reduced glutathione (GSH)	11.7	4.85
2-mercaptoethanol (ME)	13.2	5.60
N-acetylcysteine (*N*-AcCySH)	15.5	6.75
4′-phosphopantetheine (4′-PP)	21.7	9.85
pantetheine (P)	23.5	10.75

[a] The void volume of the column was measured with SBD-Cys as a marker under elution with 100% CH_3CN (retention time of SBD-Cys 2.0 min).

was adopted from 100% solvent A (0.15 M H_3PO_4–CH_3CN (97:3)) to 100% solvent B (0.15 M H_3PO_4–CH_3CN (80:20)) over 30 min. This gradient was able to separate eight SBD derivatives within 24 min (Table I). The detection limits (signal to noise ratio of 2) under these HPLC conditions were in the range 0.09 pmol (SBD-homoCys) to 0.63 pmol (SBD-Cys).

When grapefruit pulp without irradiation was derivatized with SBD-F at 60 °C and pH 9.3 for 60 min, GSH (retention time 11.7 min) was obtained as the main thiol (Figure 2A). The minor peaks corresponding to cysteine (CySH, retention time 4.7 min), cysteinylglycine (CySGly, retention time 7.0 min), and a few unknown peaks (eluted at about 2.0, 2.7, 3.0, 4.0, and 4.2 min, etc.) also appeared on the chromatogram. The peaks eluting at about 18.5, 20.5, 27.5, and 29.5 min were those corresponding to endogeneous compounds in grapefruit, because those peaks appeared on the chromatogram obtained from the blank solution without SBD-F (Figure 2B). HCySH, eluting at 8.9 min (Table I), was not contained in grapefruit pulp (Figure 2A) and so was selected as an internal standard in subsequent work.

Change of Thiol Compounds in Irradiated Pulp Sample. The changes of thiol compounds in irradiated pulp samples were investigated after various doses of irradiation. Each chromatogram for the sample irradiated at 0–1000 krad is shown in Figure 3, and degradation ratios at the different doses are in Figure 4. The amounts of CySH were about the same as those in all doses tested, and no significant differences were observed when compared with the unirradiated sample (Figure 4A). On the other hand, the peaks of both reduced glutathione (GSH) eluted at 11.7 min and unknown compound (UK) eluted at 22.0 min were exponentially decreased with the increment of the doses (Figure 4B,C). The remaining ratios of GSH and UK at 100-krad irradiation were 80% and 45%, respectively. The G value of GSH obtained from 100-krad irradiation was 0.29. However, a new peak did not appear on the chromatograms even when the sample was irradiated at 1000 krad (Figure 3). Considering the selectivity of SBD-F toward thiols (Toyo'oka and Imai, 1984), both GSH and UK must have been converted not to the compound having the SH group but to the compound without the SH group in the structure. Since its retention time is between that of 4′-phosphopantetheine (4′-PP) and pantetheine (P), the UK may be a low molecular weight thiol such as a short-chain peptide. Other endogenous peaks from the sample did not significantly change with γ-irradiation (Figure 3).

Decomposition of Authentic GSH with γ-Irradiation. The effect of γ-irradiation on authentic GSH was also investigated by HPLC with UV detection at 210 nm. As shown in Figure 5, GSH dissolved in water was converted mainly to oxidized glutathione (GSSG) with γ-irradiation. After 100- and 200-krad doses, 74% and 61%, respectively, of the original GSH remained. The yields of GSSG at 100 and 200 krad were 26% and 35%, respectively. A few small peaks eluting before GSH also appeared on the chromatogram and increased with higher doses. The stability of GSH in 0.1 M citrate buffer (pH 3), corresponding to the pH of grapefruit juice, was also tested in the same manner. Of the GSH remaining, 89% and 81% GSH after 100 and 200 krad, respectively. The yields of GSSG in the buffer solution were reduced compared to those in water, and the remaining percentages after 100 and 200 krad were 11% and 17%, respectively.

From the results of authentic GSH in water, GSH would be converted to GSSG, through the reaction of OH• with GSH to form GS•, if there were no other reactions that compete for OH• or that reduce GSSG. In pH 3 solution, however, the components of citrate buffer might be competing for OH•, because no compound except GSH and buffer components was contained in pH 3 solution. Therefore, citrate buffer components may result in a lower yields of GSSG at pH 3.

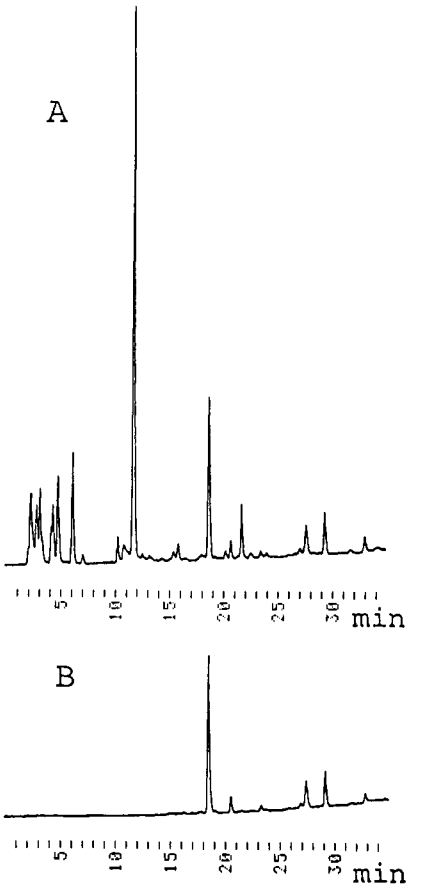

Figure 2. Chromatograms of grapefruit pulp obtained from the reaction with and without SBD-F: (A) chromatogram obtained from the reaction with SBD-F; (B) chromatogram obtained from the reaction without SBD-F. The homogenate of grapefruit pulp was centrifuged at 3500 rpm for 10 min, and 0.1 mL of the supernatant was diluted to 1.0 mL with H_2O containing 5 mM Na_2EDTA. Of the diluted solution, 0.1 mL was reacted with or without SBD-F (20 mM) at 60 °C and 0.1 M borax (pH 9.3) for 60 min. After cooling on ice–water, 20 μL of the solution was injected onto the HPLC column. HPLC conditions: column, Inertsil ODS (150 × 4.6 mm (i.d.), 5 μm); column temperature, 40 °C; eluent A, 0.15 M H_3PO_4–CH_3CN (97:3); eluent B, 0.15 M H_3PO_4–CH_3CN (80:20); gradient elution from 100% A to 100% B over 30 min and 100% B to 100% A over 5 min; flow rate, 1.0 mL/min; fluorescence detection, excitation at 380 nm, emission at 510 nm.

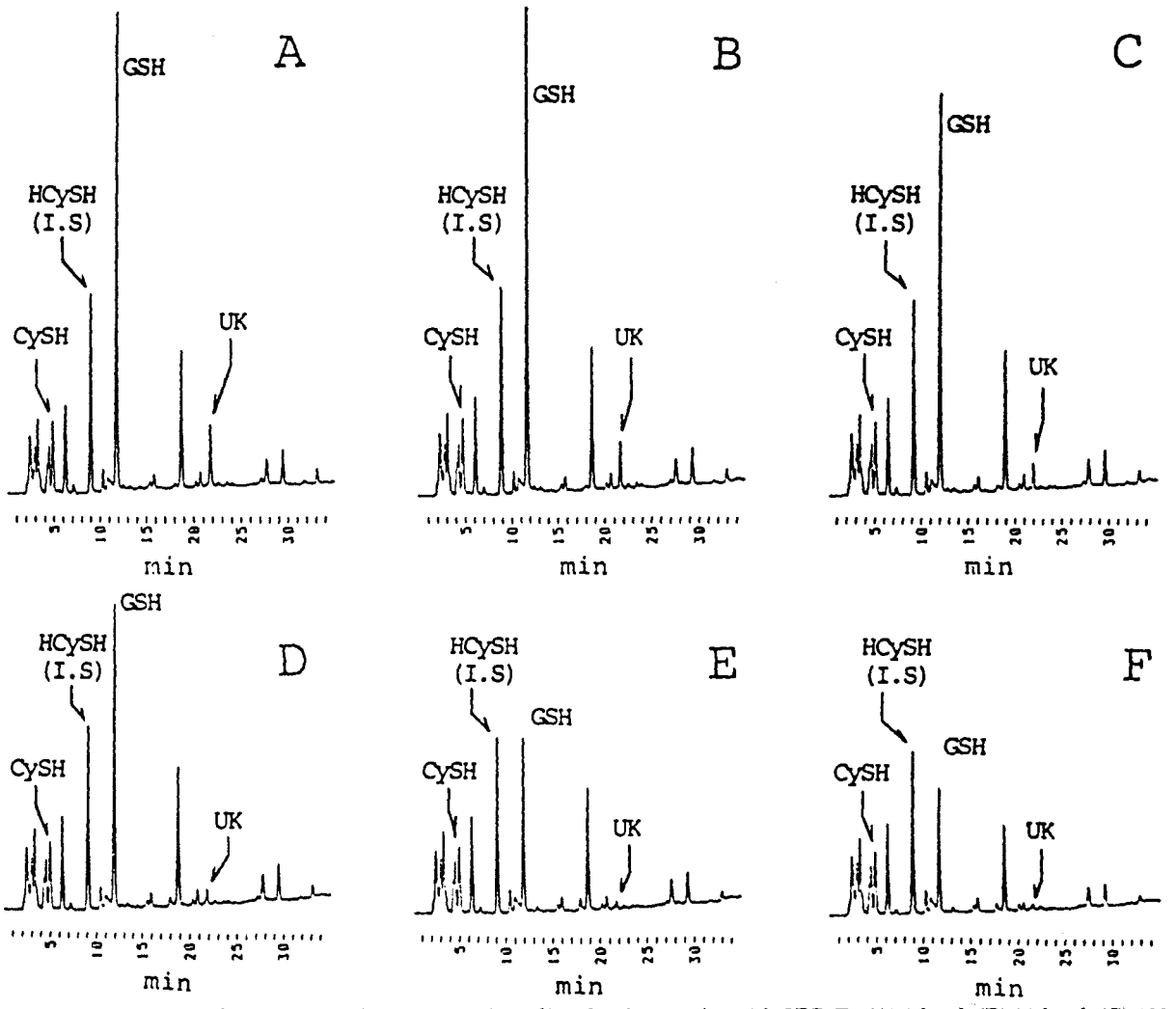

Figure 3. Chromatograms obtained from the reaction of irradiated pulp samples with SBD-F: (A) 0 krad, (B) 50 krad, (C) 100 krad, (D) 200 krad, (E) 500 krad, (F) 1000 krad. The derivatization and HPLC conditions are the same as those in Figure 2.

Table II. Concentrations of Thiols (RSH) in Unirradiated and Irradiated Grapefruits

irrad, krad	storage time, days	mean ± SD GSH, μM		CySH, μM		UK,[a] μM	
		$n = 5$	$n = 20$	$n = 5$	$n = 20$	$n = 5$	$n = 20$
0	0	143.3 ± 18.8	151.6 ± 21.3	54.3 ± 16.4	42.0 ± 11.9	8.1 ± 1.4	12.1 ± 3.8
	15	178.1 ± 13.0		35.5 ± 5.5		14.0 ± 2.9	
	30	146.4 ± 19.7		40.5 ± 9.6		10.5 ± 2.9	
	45	138.6 ± 6.2		37.8 ± 4.4		15.6 ± 2.4	
30	0	134.7 ± 12.0	133.6 ± 15.6*[b]	45.8 ± 9.9	41.2 ± 8.4	10.8 ± 3.3	11.4 ± 3.1
	15	151.2 ± 10.9*		44.6 ± 7.4		11.6 ± 2.8	
	30	130.8 ± 6.4		36.0 ± 7.4		11.1 ± 3.6	
	45	117.5 ± 11.3*		37.6 ± 2.9		12.0 ± 3.4*	
50	0	128.7 ± 10.4	129.0 ± 15.3*	46.6 ± 5.4	44.8 ± 10.9	9.3 ± 3.0	10.7 ± 2.5
	15	144.6 ± 16.2*		58.1 ± 11.6*		12.8 ± 1.4	
	30	127.5 ± 8.7*		38.1 ± 3.9		10.3 ± 2.2	
	45	115.2 ± 11.3*		36.3 ± 4.0		10.3 ± 2.2*	
100	0	125.5 ± 13.6	123.0 ± 18.9*	51.5 ± 12.1	45.5 ± 9.6	9.1 ± 2.6	10.4 ± 2.7
	15	147.7 ± 8.0*		45.9 ± 6.8		12.7 ± 2.3	
	30	113.5 ± 10.8*		43.6 ± 7.8		10.2 ± 2.7	
	45	105.4 ± 7.2*		40.9 ± 10.4		9.8 ± 2.4*	
200	0	117.0 ± 14.0*	97.7 ± 18.5*	51.5 ± 11.0	53.8 ± 12.1*	7.9 ± 1.0	7.8 ± 2.0*
	15	107.0 ± 8.7*		58.9 ± 6.7*		8.9 ± 2.3*	
	30	89.9 ± 11.7*		41.8 ± 4.2		7.8 ± 2.6	
	45	77.1 ± 5.7*		63.2 ± 13.3*		6.5 ± 1.8*	

[a] The values were calculated based on the calibration curve of GSH. [b] $p < 0.01$ when compared to control (0-krad) value.

Determination of Thiol (RSH) and Disulfide (RSSR) in Unirradiated and Irradiated Grapefruits. When the area ratios of SBD derivatives to SBD-homoCys (internal standard) were plotted against the concentration of thiols or disulfides, linearity was observed and correlation coefficients (γ) of the calibration curves were greater than 0.998. Therefore, the proposed method was thought to be suitable for the determination of thiols and disulfides.

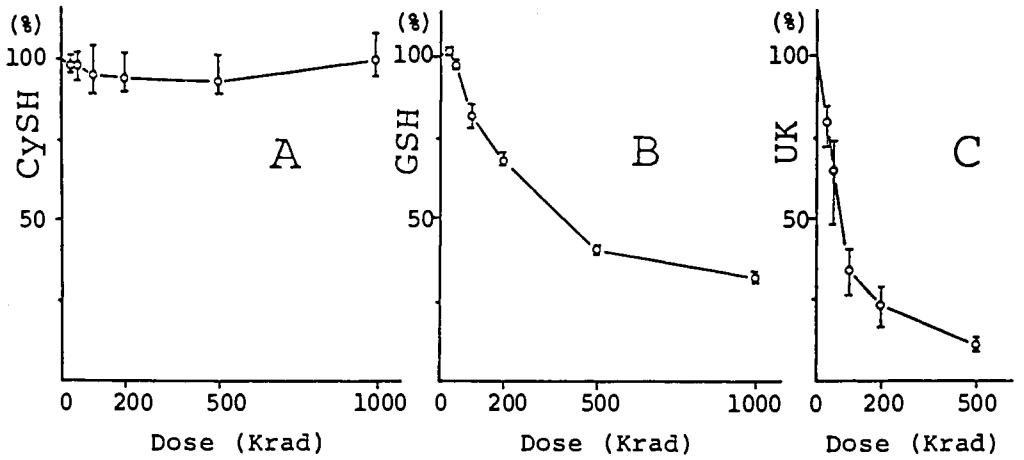

Figure 4. Degradation curves of thiol compounds at various doses: (A) CySH, (B) GSH, (C) UK. The value obtained from unirradiated sample was arbitrarily taken as 100% (n = 5).

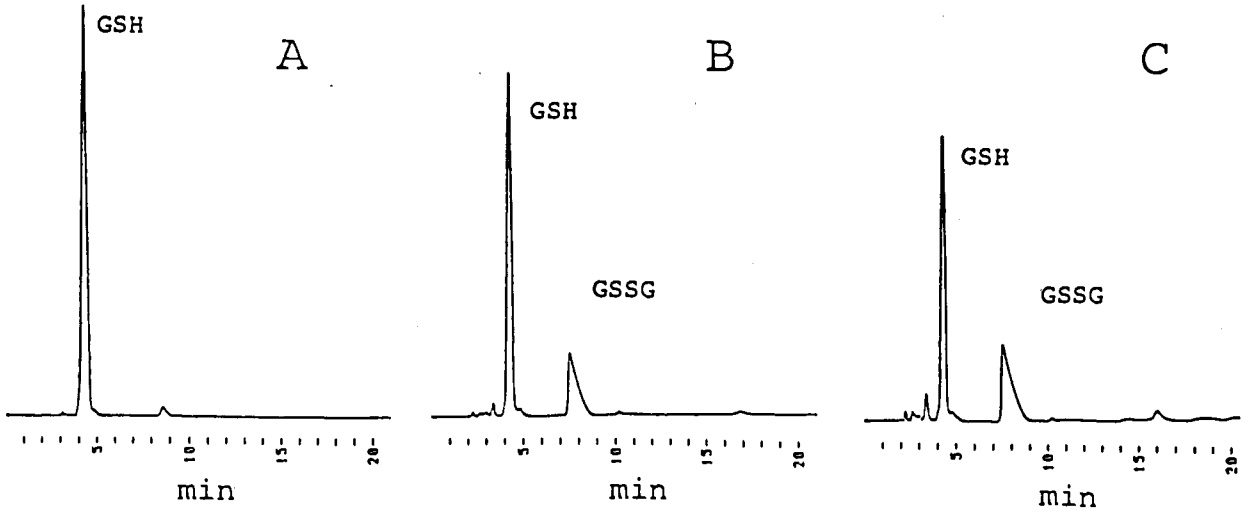

Figure 5. Chromatograms obtained from γ-irradiation of authentic GSH in water: (A) 0 krad, (B) 100 krad, (C) 200 krad. Concentration of GSH 1.1 mM. HPLC conditions: isocratic elution, H_2O; detection, UV 210 nm. Other HPLC conditions are the same as those in Figure 2.

Table III. Concentrations of Disulfides (RSSR) in Unirradiated and Irradiated Grapefruits

		mean ± SD			
		CySSCy, μM		$(UK)_2$,[a] μM	
irrad, krad	storage time, days	n = 5	n = 20	n = 5	n = 20
0	0	4.3 ± 1.8	5.5 ± 3.0	16.3 ± 2.4	13.7 ± 3.9
	15	8.9 ± 3.4		16.4 ± 3.6	
	30	4.8 ± 1.7		12.9 ± 2.6	
	45	4.1 ± 1.7		9.4 ± 1.3	
30	0	8.2 ± 3.1	6.7 ± 3.5	19.5 ± 5.0	14.0 ± 5.0
	15	8.8 ± 3.8		13.6 ± 4.7	
	30	6.2 ± 1.0		13.1 ± 1.8	
	45	3.5 ± 2.6		10.1 ± 1.3	
50	0	6.9 ± 2.2	6.8 ± 3.5	17.2 ± 3.3	14.0 ± 3.8
	15	11.0 ± 3.4		16.9 ± 2.0	
	30	4.3 ± 2.1		11.5 ± 2.1	
	45	5.0 ± 0.9		10.2 ± 1.1	
100	0	6.6 ± 3.7	5.4 ± 3.5	15.9 ± 2.6	12.1 ± 3.7
	15	6.7 ± 4.3		14.5 ± 2.3	
	30	3.6 ± 1.1		9.4 ± 1.1	
	45	4.7 ± 2.0		8.6 ± 0.9	
200	0	7.1 ± 3.5	6.4 ± 4.0	13.8 ± 1.4	10.5 ± 2.9
	15	10.4 ± 3.9		11.7 ± 2.0	
	30	4.6 ± 1.9		7.4 ± 1.5	
	45	3.6 ± 2.5		9.2 ± 1.5	

[a] The values were calculated based on the calibration curve of GSSG.

As listed in Table II, the average concentrations of CySH, GSH, and UK in unirradiated grapefruits (n = 20) were 42.0, 151.6, and 12.1 μM (this value was tentatively calculated based on the calibration curve of GSH), respectively. Similar values of CySH were obtained from irradiated and unirradiated grapefruits. The concentrations

of GSH gradually and significantly decreased with increased dose ($p < 0.01$) (Table II). The degradation ratios of GSH were similar to those of irradiated pulp samples. However, the decomposition ratios of UK in the irradiated grapefruits (Table II) were considerably lower than those from irradiated pulp samples. The higher decomposition in irradiated pulp samples might be due to oxygen dissolved in the homogenate before irradiation.

Determinations of oxidized form (RSSR type), crystine (CySSCy), GSSG, and $(UK)_2$ were performed by subtraction of reduced form (RSH type) from total thiols (RSH, RSSR). Tri-*n*-butylphosphine was used for the reduction of RSSR to RSH (Humphrey and Potter, 1965; Kawahara et al., 1981), since the reduction with TBP yields quantitatively 2 mol equiv of RSH in slightly alkaline medium and does not react with SBD-F. The concentration of GSSG in grapefruit pulp was extremely low (1–5 μM) and did not increase with dose (data not shown). The average concentrations of CySSCy and $(UK)_2$ were 5.5 and 13.7 μM (this value was tentatively calculated based on the calibration curve of GSSG), respectively (Table III), and were independent of dose. Therefore, thiol compounds (RSH) in grapefruit pulp were not converted to disulfides (RSSR), but to other compounds lacking in SH group.

When the model fruit is exposed to radiation, most of the radiation energy is absorbed by the water, then forming $OH^{\bullet}$ (Basson et al., 1983). The $OH^{\bullet}$ must react with many other compounds (sugars, lipids, etc.) present in the grapefruit pulp to generate other organic radicals, $R^{\bullet}$. The $OH^{\bullet}$ also reacts with low concentration of GSH in the pulp to generate $GS^{\bullet}$. However, the fate of $GS^{\bullet}$ thus produced is difficult to predict in a complex matrix like grapefruit, since the concentration of GSSG, which is formed from dimerization of $GS^{\bullet}$, was lower.

The concentration changes on thiols and disulfides at various storage period (0–45 days) were also examined. The amount of GSH reached maximum value at 15 days after irradiation and subsequently decreased with storage period (Table II). On the other hand, the concentrations of CySH, UK, CySSCy, and $(UK)_2$ were about the same as those in all grapefruits stored at 10 °C, although slight decrements were observed (Tables II and III).

CONCLUSION

The proposed method for the determination of thiol compounds in grapefruit is also applicable to the determination in other foods due to its simplicity, selectivity, and sensitivity. However, the determination of thiols was not useful for identifying irradiated grapefruit. The main limitations are the variability of GSH in grapefruit, the low loss at doses below 100 krad, and the instability of GSH during storage period.

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Inhibition of the Catecholase Activity of Mushroom Tyrosinase by Carbon Monoxide

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The catecholase activity of tyrosinase extracted from freeze-dried mushroom powder was found to be reversibly inhibited by carbon monoxide. Dissociation of the carbon monoxide–enzyme complex was followed at various pHs between 4.0 and 8.0. The minimum value of the first-order rate constant for this reaction coincides with the pH of the maximum catecholase activity pH 6.5. Carbon monoxide treatment of the enzyme extracts prevented the self-inactivation of the enzyme.

Tyrosinase (monophenol, dihydroxyphenol oxidoreductase EC 1.14.18.1) is a copper-containing enzyme that catalyzes two reactions in which molecular oxygen is the hydrogen receptor, phenol is the hydrogen donor, and AH_2 represents the hydrogen donor:

$$\text{monophenol} + O_2 + AH_2 \rightleftharpoons o\text{-dihydroxyphenol} + H_2O + A$$

$$o\text{-dihydroxyphenol} + \tfrac{1}{2}O_2 \rightleftharpoons o\text{-quinone} + H_2O$$

A number of substances are known to inhibit this enzyme. They can be conveniently divided into three types (Robb, 1985): (1) general chelating agents for copper, (2) noncompetitive inhibitors with respect to the phenolic substrate, and (3) analogues of phenols, although a clear distinction is not always possible. Carbon monoxide a known inhibitor of many copper-containing oxidases and can be expected to act as an inhibitor of type 2 above.

Although carbon monoxide has been suggested as an inhibitor for tyrosinase, there are no detailed data on the subject. A gaseous inhibitor for tyrosinase could have an important role in maintaining the quality of fresh mushrooms and other vegetables during storage. The objective of this work was therefore to investigate the effect of carbon monoxide on mushroom tyrosinase activity.

RESULTS AND DISCUSSION

The pH dependence of the catecholase activity of the extracted tyrosinase was investigated over the range pH 4.5–9 using tyrosinase citrate–phosphate buffers. The specific activity of the extracted tyrosinase at pH 4.0 was 0.2 (μmoles/minute per milligram of protein), which increased to a maximum of 1.7 at pH 6.5 (Figure 1). This activity profile is similar to those previously reported (McCord and Kilara, 1983). The tyrosinase activity of the carbon monoxide treated extract was considerably reduced compared to the control, 0.034 and 0.55, respectively, while the nitrogen-treated aliquot was only slightly reduced 0.45. Since the activity of the nitrogen-treated extract was considerably higher than the carbon monoxide treated extract, the effect of the carbon monoxide is not simply due to reducing the dissolved oxygen concentration. Carbon monoxide has been shown to be a inhibitor for many copper-containing oxygenases. The effect of carbon monoxide on tyrosinase was found to be reversible; the activity of the carbon monoxide treated extract was restored to that of the control after bubbling through air. The kinetics of the tyrosinase reactivation was studied by following the enzyme activity when a carbon monoxide saturated extract was mixed with buffer saturated with air in the absence of substrate. Various pHs in the range 4.0–8.0 were investigated. In each case, after an initial rapid increase the activity approached asymtopically the maximum value (Figure 2). If the reactivation is assumed to follow first-order kinetics, then it can be described by the equation $A_t = A_m(1 - e^{-kt})$, where A_t is the activity at time t, A_m is the initial activity, and k is the reaction rate constant. A linear relationship was found between ln $(1 - A_t/A_m)$ and t for the reactivation at all pHs, from which the reaction rate constants were calculated (Figure 1). It can be seen that the smallest value of k coincides with the pH maximum activity, pH 6.5. Tyrosinase has been shown to be subject to substrate-induced inactivation. The transformed substrate becomes covalently attached to the enzyme blocking the entry of fresh substrate (Schwimmer, 1981). Our experiments show that this self-inactivation can be prevented by carbon monoxide. Both carbon monoxide treated and untreated enzyme preparations were incubated in the presence of catechol. Aliquots were taken and mixed with fresh substrate and the activity assayed. The carbon monoxide treated solution retained its activity until at 17 min excess air was introduced by vigorous stirring and thus carbon monoxide removed (Figure 3). With the untreated enzyme extract, a steady drop in ac-

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