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Effects of Lipase, Lipoxygenase, Peroxidase, and Rutin on Quality Deteriorations in Buckwheat Flour

TATSURO SUZUKI,* YUTAKA HONDA, YUJI MUKASA, AND SUN-JU KIM

Department of Upland Agriculture, National Agricultural Research Center for the Hokkaido Region, Shinsei, Memuro, Kasai-gun, Hokkaido 082-0071, Japan

To investigate the effects of changes in lipase, lipoxygenase, peroxidase (POX), and rutin concentrations on the quality of buckwheat flour, 14 buckwheat varieties were stored for 0, 4, 10, and 30 days at 5 or 20 °C. During the storage period, lipase activity correlated to pH (significantly negative) and water-soluble acid (WSA) (significantly positive). The lipoxygenase 1 protein concentration had a negative correlation to WSA (significant at 0 and 4 storage days at 5 °C and at 0 and 10 storage days at 20 °C). POX had significant correlation to pH and peroxide value (POV) at 5 °C, whereas it was not significant at 20 °C. The rutin concentration had negative correlations to WSA (significant at 30 days of storage at 5 °C and at 4 days of storage at 20 °C). Thus, lipase activity plays an important role that relates to lipid degradation in quality deterioration of buckwheat flour.

KEYWORDS: Lipase; lipoxygenase; peroxidase; rutin; deterioration; buckwheat flour; fatty acid

INTRODUCTION

In Japan, buckwheat (*Fagopyrum esculentum* Moench) flour is used mainly for making noodles. Japanese buckwheat noodle makers value the freshness of buckwheat flour very much. However, buckwheat flour deteriorates easily (*1*, *2*). Several studies have shown that lipid degradation and oxidation in buckwheat flour are the main changes in measurable indices of quality deterioration during storage. Therefore, understanding lipid degradation pathways is important to understanding the quality deterioration mechanisms in buckwheat flour. Pathways of lipid degradation and oxidation have been well studied in rice and soybean (*3*; **Figure 1**). According to these studies, lipase (triacylglycerol lipase EC 3.1.1.3) (LIP), lipoxygenase (EC 1.13.11.12) (LOX), and peroxidase (EC 1.11.1.7) (POX) are important in quality deterioration.

LIP catalyzes the first step of lipid catabolism. LIP is an important concern in the food industry because lipid hydrolysis can cause deterioration of food quality (*4*). LOX is thought to have a significant effect on quality in soybeans (*5*, *6*) and rice (*7*). POX also plays roles in food quality, including deterioration of color and flavor (*4*). In soybeans, carbonyl compounds such as aldehydes and ketones are the major contributors to "beany" and "green" flavors. They are mainly generated by lipid peroxidation, and enzyme activities such as POX together with LOX play important roles (*8*−*10*).

Several studies have shown that enzymatic activities play important roles in quality deterioration in buckwheat flour (*11*−*14*), and LIP and POX have been characterized (*11*, *12*, *15*) (POX has been partially characterized). Ohinata proposed that accumulation of free fatty acids is mainly caused by LIP activity in buckwheat flour during storage (*15*). An increase in free fatty acids indicates deterioration of the quality of buckwheat flour (e.g., increase of water-soluble acid) and will result in lipid peroxidation and deterioration of the flavor. However, the effects of LOX and POX on quality deterioration have not been investigated. In addition, which enzymes (LIP, LOX, or POX) are important for quality deterioration in buckwheat flour has not been clarified. To elucidate this, we stored 14 varieties of

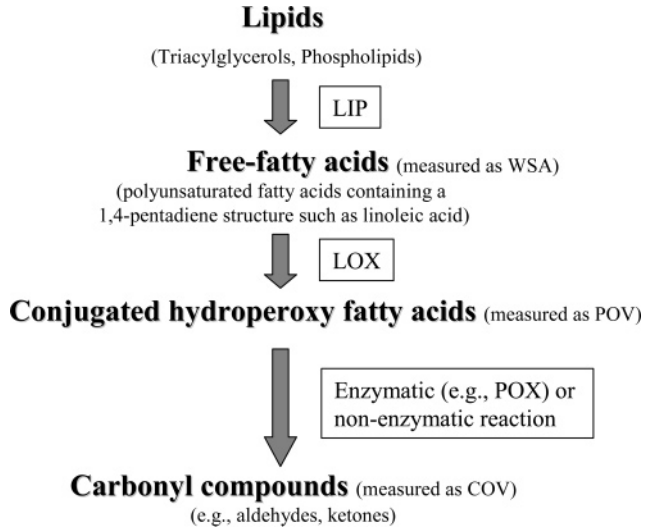

Figure 1. Model of lipid degradation proposed for rice bran. LIP, lipase; WSA, water-soluble acid; LOX, lipoxygenase; POV, peroxide; POX, peroxidase; COV, carbonyl value.

* Address correspondence to this author at the Genetic Resources Laboratory, Department of Upland Agriculture, National Agricultural Research Center for the Hokkaido Region, Shinsei, Memuro, Kasai-gun, Hokkaido, 082-0071 Japan (e-mail tsuzu@affrc.go.jp; telephone +81-155-62-9273;fax +81-155-61-2127).

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Table 1. Rutin, LOX1, and LOX2 Concentrations and POX and LIP Activities of Examined Varieties[a]

ID	variety	rutin (mg/100 g of flour)	LIP relative activity[b] (%)	LOX1 relative content[c] (%)	LOX2 relative content[c] (%)	POX relative activity[d] (%)
1	Kitawasesoba	0.337	43	69	66	38
2	Kmx1	0.303	52	94	47	65
3	Kmx2	0.390	67	100	52	45
4	Sumchanka	0.231	59	93	65	38
5	Km8	0.311	48	86	64	38
6	HC10-7	0.186	75	47	42	67
7	HC10-11	0.257	68	68	67	70
8	HC10-21	0.190	78	94	66	91
9	HC10-23	0.222	73	71	65	55
10	Tanno/hiushinai 38	0.253	64	88	69	33
11	Tanno/hiushinai 39	0.239	66	14	20	50
12	Tanno/hiushinai 43	0.246	95	53	50	100
13	Km6	0.346	100	67	58	52
14	Amasoba	0.064	83	38	30	39

[a] Data are means of two independent experiments.The two measurements did not differ from average by more than 4.0% (rutin), 8.8% (LOX 1), 10.1% (LOX 2), 12.3% (LIP), or 6.6% (POX). [b] ID 13 = 100. [c] ID 3 of LOX1 = 100. [d] ID 12 = 100.

buckwheat flour (for 0, 4, 10, and 30 days at 5 or 20 °C) and investigated the effects of changes of LIP, LOX, and POX on quality deterioration in buckwheat flour.

MATERIALS AND METHODS

Plant Materials. Common buckwheat varieties were grown at the experimental field of the National Agricultural Research Center for the Hokkaido region in Memuro, Hokkaido, Japan (longitude, 143° 03′; latitude, 42° 53′). Buckwheat seeds were sown on June 6, 2001, and harvested in late August. Harvested seeds were dried, threshed, and dehulled. Dehulled seeds were milled with a stone grinder and immediately used for storage tests.

Storage Test. Before the storage test, we screened 14 of 46 buckwheat varieties to obtain a wide range variation of LIP activity, POX activity, LOX protein concentration, and rutin concentration. Buckwheat flour was placed in polyethylene bags and stored at 5 or 20 °C in a dark room for 0, 4, 10, and 30 days (0 storage days means immediately after milling). Then, samples were stored in aluminum laminated bags with an oxygen absorber at −130 °C until analysis.

Lipid Extraction. Lipid was extracted according to the method of Folch (*16*). One gram of buckwheat flour was mixed with 10 mL of chloroform/methanol (2:1, v/v) (CM solution) at 37 °C for 60 min. A crude extract was obtained by centrifugation, and the extract was washed three times with deionized distilled water. The chloroform layer was concentrated with a vacuum evaporator. Lipids were dissolved in 1 mL of CM solution, and then the peroxide value (POV) and carbonyl value (COV) were measured immediately.

Determination of pH, WSA, POV, and COV. One gram of buckwheat flour was mixed with 10 mL of deionized distilled water, and the pH was measured. The water-soluble acid (WSA) was measured by titration using KOH according to the official method of the Japan Oil Chemists' Society. The POV was measured by titration using a 100 mM KOH (ethanol solution) according to the official method of the Japan Oil Chemists' Society. The total carbonyl compounds in total lipids were estimated by measuring the COV according to the method of Kumazawa (*17*).

Rutin Concentration. Buckwheat flour was homogenized in 1 mL of methanol/0.1% (v/v) phosphoric acid (9:1) per 100 mg of fresh weight and extracted at 80 °C for 3 h. The centrifuged supernatant was passed through a filter and analyzed by HPLC according to the method of Suzuki et al. (*18*).

Preparation of Crude Protein Extract. One hundred milligrams of buckwheat flour was mixed with 1 mL of 50 mM acetate−NaOH buffer (pH 5.0) for 20 min on ice. A crude protein extract was obtained by centrifugation at 4 °C and used immediately.

Assay of in Vitro POX Activity. Prior to the assay of the POX activity, we purified and characterized POX in buckwheat flour: substrate specificity (K_m value), pH stability, thermal stability, optimal pH, and optimal temperature were determined. POX activity was determined spectrophotometrically according to the modified procedure of Amako (*19*) and Yamasaki (*20*) using *o*-dianisidine as a substrate. POX activities were determined at 22 °C by measuring the initial rate of increase in absorbance at 430 nm. The assay mixture contained 0.05 mM substrate, 200 mM acetate−NaOH buffer (pH 5.0), and 50 μL of crude protein extract in a total volume of 0.3 mL. The assay was initiated by the addition of the enzyme.

Assay of in Vitro LIP Activity. LIP activity was determined spectrophotometrically according to the procedure of Suzuki (15) using *p*-nitrophenyl laurate (*p*NPC12) as a substrate. The assay mixture contained 0.5 mM *p*NPC12 in a 200 mM acetate−NaOH buffer (pH 6.0) containing 0.3% Triton X-100, 4% (v/v) acetone, and 50 μL of crude protein extract in a total volume of 0.3 mL. The assay was initiated by the addition of the enzyme. Activities were determined at 22 °C by measuring the initial rate of increase in absorbance at 400 nm.

LOX Protein Concentration. It is difficult to assay in vitro LOX activity in buckwheat seeds (*21*). Thus, we performed immunoblotting analysis with a LOX-specific antibody to determine the LOX protein concentration.

Preparation of Anti-LOX Antibody. A polyclonal antibody raised against soybean LOX was prepared using the purified soybean LOX (a mixture of three LOX isozymes: LOX1, LOX2, and LOX3). Soybean LOX was purified to homogeneity using $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, gel filtration chromatography, and preparative native polyacrylamide gel electrophoresis (PAGE). Two rat pups were used as the immune animals. After preparation of immune sera, anti-LOX-IgG was affinity purified with the antigen according to the method of Yamaya (*22*).

Electrophoresis and Electroblotting. SDS-PAGE was carried out using 6% polyacrylamide gels (*23*). Electroblotting was carried out using a tank blotting apparatus (Bio-Rad) with a minor modification (electroblot for 14 h at 30 mV at 4 °C) according to the method of Hayakawa (*24*).

Immunoblotting Analysis of LOX Protein. Immunoblotting was performed with the affinity-purified LOX specific IgG. The immunoreacted protein was incubated with a goat anti-rat IgG horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and visualized with an ECL+Plus detection kit (Amersham Pharmacia Biotech) on X-ray film (X-OMAT, Kodak). The intensities of LOX1 (higher molecular weight) and LOX2 (lower molecular weight) were quantitated densitometrically. We have confirmed that this antibody is monospecific to LOX protein (data not shown).

Effects of Rutin Concentration against in Vitro LIP Activity. To investigate the inhibitory effect of rutin against buckwheat LIP activity, rutin was added to a reaction mixture, and the LIP activity was measured

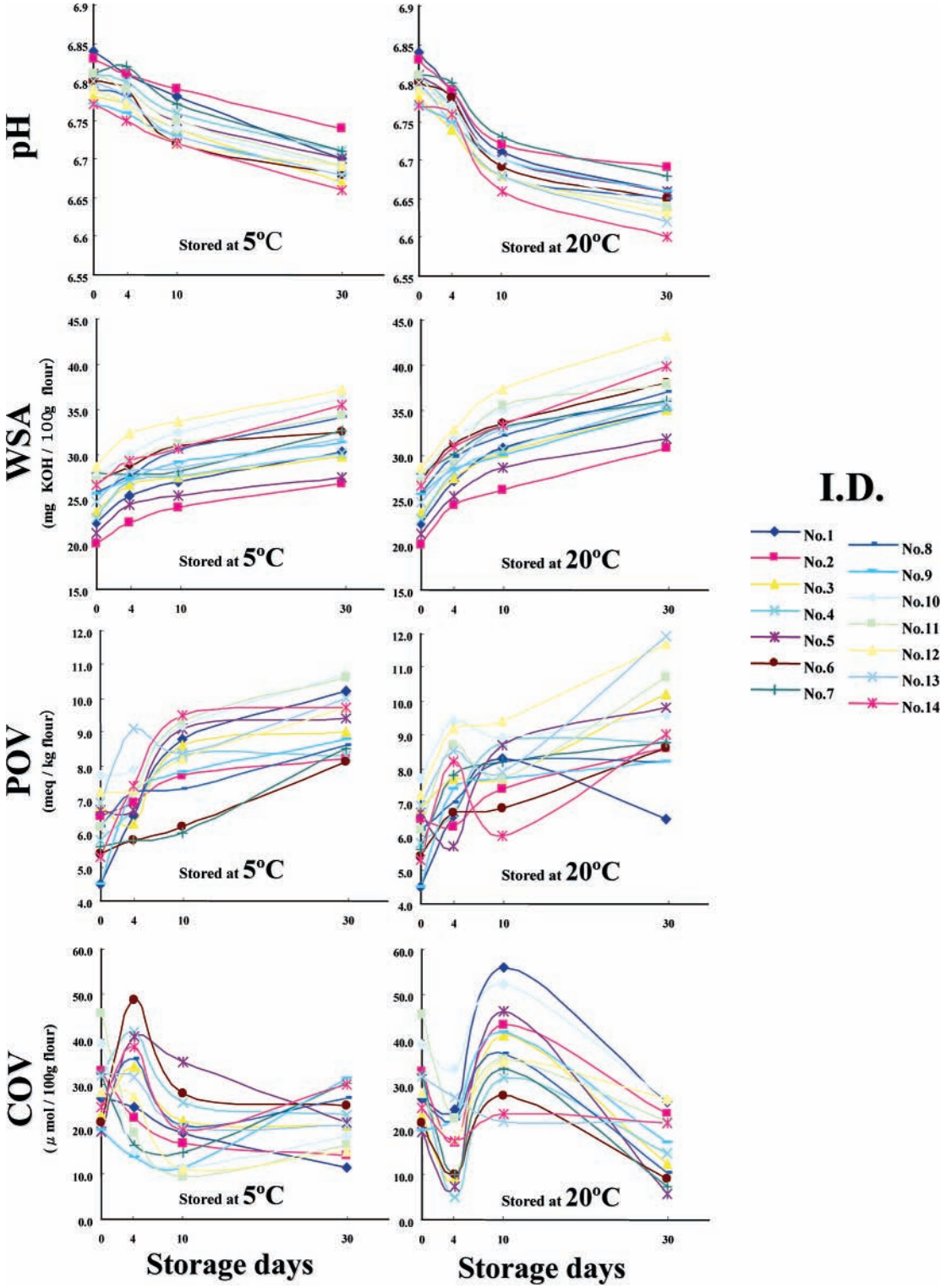

Figure 2. Changes in pH, WSA, POV, and COV of 14 buckwheat varieties during storage. WSA, water-soluble acid; COV, carbonyl value; POV, peroxide value. Data are means of two independent experiments. ID numbers are the same as in **Table 1**. The storage test was performed at 5 or 20 °C using freshly milled buckwheat flour. The two measurements did not differ from the average by more than 4.2% (pH), 6.2% (WSA), 8.3% (POV), or 9.4% (COV).

(described above). As a substrate, *p*NPC12 for LIP activity was used. Rutin was dissolved in 2-methoxyethanol and added to the reaction mixture with a final 2-methoxyethanol concentration of 15% (v/v) and a final rutin concentration of 0−200 μg/mL. The reaction mixture contained LIP activity corresponding to 66.7 mg of buckwheat flour per 1 mL of reaction mixture.

Statistics. The correlation matrix (Pearson correlation coefficient) was calculated by using the Microsoft Excel program.

RESULTS AND DISCUSSION

LIP and POX Activities, LOX1 and LOX2 Protein Concentrations, and Rutin Concentration of Varieties Examined. Table 1 shows the rutin, LOX1, and LOX2 concentrations and POX and LIP activities of 14 varieties used in the storage test. The rutin concentration ranged from 0.064 to 0.390 mg/100 g, the LOX1 concentration from 100 to 14.2% (relative

Table 2. Correlation Matrix of Values for Measurements of Rutin, LIP, LOX, POX, pH, WSA, COV, and POV Stored at 5 or 20 °C

		5 °C					20 °C				
		rutin	LIP	LOX1	LOX2	POX	rutin	LIP	LOX1	LOX2	POX
pH	0 days	0.45[a]	**–0.62**[b]	0.09	0.12	–0.10	0.45	**–0.62**	0.09	0.12	–0.10
	4 days	0.39	**–0.55**	0.16	0.23	0.04	0.01	**–0.57**	0.05	0.22	0.01
	10 days	0.46	**–0.77**	0.32	0.22	-0.25	0.38	**–0.61**	0.39	**0.54**	–0.04
	30 days	0.34	**–0.57**	0.33	0.24	0.06	0.35	**–0.66**	0.45	0.43	0.12
	4–0 days	–0.14	0.14	0.16	0.24	0.32	**–0.64**	0.19	–0.07	0.10	0.16
	10–4 days	0.20	–0.48	0.31	0.03	–0.50	**0.57**	–0.12	0.52	0.50	–0.07
	30–10 days	–0.31	0.51	–0.06	–0.02	**0.56**	0.11	–0.39	0.32	–0.01	0.33
WSA	0 days	–0.50	**0.64**	**–0.56**	–0.21	0.38	–0.50	**0.64**	**–0.56**	–0.21	0.38
	4 days	–0.45	**0.71**	**–0.54**	–0.25	0.29	**–0.55**	**0.66**	–0.45	–0.15	0.37
	10 days	–0.51	**0.63**	–0.51	–0.26	0.34	–0.35	**0.60**	**–0.61**	–0.29	0.29
	30 days	**–0.55**	**0.63**	–0.52	–0.24	0.33	–0.50	**0.61**	–0.48	–0.25	0.30
	4–0 days	0.23	0.01	0.18	–0.03	–0.29	0.03	–0.15	0.46	0.24	–0.16
	10–4 days	–0.37	–0.03	–0.10	–0.11	0.27	0.24	0.16	**–0.57**	–0.39	–0.02
	30–10 days	–0.35	0.27	–0.23	–0.06	0.10	–0.50	0.19	0.18	0.03	0.10
POV	0 days	0.32	0.23	0.26	0.01	0.12	0.32	0.23	0.26	0.01	0.12
	4 days	0.02	0.52	–0.07	–0.02	–0.10	–0.15	**0.62**	–0.41	–0.26	0.12
	10 days	0.07	–0.14	–0.12	–0.25	**–0.60**	0.45	–0.14	0.39	**0.61**	0.17
	30 days	0.12	0.08	–0.40	–0.19	–0.37	0.20	**0.62**	–0.24	–0.32	0.17
	4–0 days	–0.30	0.22	–0.31	–0.03	–0.20	–0.42	0.42	**–0.63**	–0.27	0.02
	10–4 days	0.06	**–0.60**	–0.07	–0.25	**–0.58**	0.42	**–0.61**	**0.60**	**0.62**	0.01
	30–10 days	0.04	0.31	–0.31	0.14	0.45	–0.07	**0.67**	–0.45	**–0.66**	0.07
COV	0 days	0.04	–0.01	–0.28	–0.28	–0.09	0.04	–0.01	–0.28	–0.28	–0.09
	4 days	–0.19	0.10	0.07	–0.14	–0.02	–0.01	0.23	–0.16	0.04	–0.05
	10 days	0.10	–0.26	0.33	0.18	–0.25	0.45	**–0.74**	0.42	0.45	–0.26
	30 days	**–0.57**	0.32	–0.03	0.11	0.08	0.03	0.14	–0.25	–0.23	–0.10
	4–0 days	–0.15	0.07	0.17	0.03	0.03	–0.04	0.23	0.09	0.29	0.03
	10–4 days	0.46	–0.47	0.27	0.46	–0.25	0.41	**–0.82**	0.49	0.37	–0.19
	30–10 days	–0.48	0.44	–0.29	–0.07	0.26	–0.38	**0.75**	**–0.54**	**–0.55**	0.16

[a] Normal type, not significant. [b] **Boldface type**: significant at 5% level. [c] **Bold and underlined**: significant at 1% level.

content), the LOX2 concentration from 68.9 to 19.8% (relative content), the POX activity from 100 to 37.7% (relative activity), and the LIP activity from 100 to 42.9% (relative activity). The range of LIP activity was narrower than those of the other components. There are some reports that investigate rutin concentration in buckwheat varieties [rutin concentration of 27 buckwheat variety ranged from 0.126 to 0.359 mg/100 g (*25*)]. Compared to that study, the range of rutin concentration in this experiment is enough wide.

Changes in pH, WSA, POV, and COV during Storage Period. Figure 2 shows the changes in pH, WSA, POV, and COV of 14 buckwheat varieties during storage. Data are expressed as an average of two independent experiments. During the storage periods, the pH decreased at both 5 and 20 °C. These results showed good agreement with the report of Muramatsu (*2*). The pH decreased more at 20 °C than at 5 °C; it decreased especially rapidly from 0 to 10 days of storage at 20 °C. WSA increased at both 5 and 20 °C, and it increased more at 20 °C than at 5 °C. These results also showed good agreement with the report of Muramatsu (*2*). In buckwheat flour, a decrease of pH and an increase of WSA strongly suggested an increase of free fatty acid (*1*, *12*).

Changes in POV differed among varieties and storage temperatures. At 5 °C, it generally increased quickly until the 10th day of storage and then slightly increased until the 30th storage day. On the other hand, the changing patterns of POV of each variety at 20 °C were different from those at 5 °C. POV is an index of the amount of peroxidative compounds such as conjugated hydroperoxy fatty acids, which will degrade to carbonyl compounds. Degradation of POV should depend on temperature; this may relate to the difference in the changing patterns of POV between 5 and 20 °C.

Changes in COV also differed among varieties and storage temperature. At 5 °C, the change could be roughly divided into two groups. One group had a maximum peak on the 4th storage day, and then it decreased until the 10th day. The other group did not have a peak on the 4th storage day at 5 °C. On the other hand, at 20 °C, it generally decreased until the 4th storage day, increased to a maximum at the 10th day, and then decreased to the 30th storage day. The COV is an index of the amounts of carbonyl compounds such as aldehydes or ketones (e.g., hexanal and nonanal), which are volatile compounds. The degree of generation and volatility of carbonyl compounds would depend on the temperature; this may be related to the difference in the changing patterns of COV between 5 and 20 °C.

Relationships among LIP and POX Activities, LOX1 and LOX2 Protein Concentrations, Rutin Concentration, pH, WSA, COV, and POV. Table 2 shows the correlation matrix among LIP and POX activities, LOX1 and LOX2 protein concentrations, rutin concentration, pH, WSA, COV, and POV. During storage at both 5 and 20 °C, LIP activity had significant negative correlations to pH (0, 4, 10, and 30 storage days) and significant positive correlations to WSA (0, 4, 10, and 30 storage days). A decrease of pH and an increase of WSA indicate deterioration of buckwheat flour. Therefore, LIP activity plays important roles in quality deterioration in buckwheat flour related to lipid degradation. Even at 0 storage days (just after milling), LIP activity had significant correlations to pH and WSA. This indicates that LIP activity had generated free fatty acids in buckwheat seed prior to milling. The pH of buckwheat flour is generally ~6.8. LIP activity should be increased by the progression of fatty acid release, which would decrease the pH, because LIP activity becomes much higher below pH 6.0 (*18*). The optimum temperature of buckwheat LIP was 30 °C (*18*),

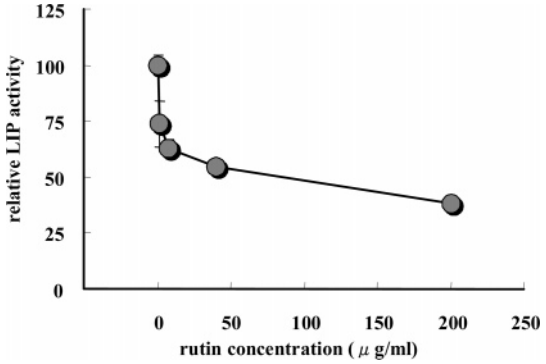

Figure 3. Inhibitory effect of rutin concentration against in vitro LIP activity. To the investigate inhibitory effect of rutin against buckwheat LIP activity, rutin was added into a reaction mixture, and the LIP activity was measured. As a substrate, *p*NPC12 was used. Rutin was dissolved in 2-methoxyethanol and added to reaction mixture with a final 2-methoxyethanol concentration of 15% (v/v) and a final rutin concentration of 0–200 μg/mL. Reaction mixture contains LIP activity corresponding to 66.6 mg of buckwheat flour per 1 mL of reaction mixture. Relative LIP activity is expressed as activity at 0 μg/mL of rutin concentration = 100. Data are means of three independent experiments. Bar indicates ± SD.

and it had ~50% of the activity at 10 °C as at 30 °C (*18*). These results are consistent with results that the pH decreased more and WSA increased more at 20 °C than at 5 °C. The LIP activity also had significant correlations to POV [4, 30, 10−4 (calculated from the value of the 10th storage day by subtracting the value of the 4th storage days) and 30−10 storage days], and COV (10, 10−4, and 30−10 storage days) at 20 °C. It indicates that LIP activity is an important factor in quality deterioration because it affects not only generation of free fatty acids but also subsequent fatty acid oxidation and degradation.

The LOX1 protein concentration had negative correlations to WSA (significant; 0 and 4 storage days at 5 °C and 0 and 10 storage days at 20 °C). LOX catalyzes the oxidation of polyunsaturated fatty acids containing a 1,4-pentadiene structure, such as linoleic acid, to conjugated hydroperoxy fatty acids. Conjugated hydroperoxy fatty acids degrade more quickly than polyunsaturated fatty acids to lower molecular weight compounds. In other words, LOX plays a role in decreasing WSA, which is an index of the amount of free fatty acids. This may relate to the negative correlations of LOX1 to WSA. On the other hand, the concentration of LOX2 protein did not significantly correlate to any index at 5 °C, whereas it significantly correlated at 20 °C with pH (10th storage day), POV (10, 10−4, and 30−10 storage days) and COV (30−10 storage day).

The effects of POX on pH, WSA, POV, and COV were not observed at 20 °C. On the other hand, at 5 °C, POX had a significant correlation to pH (30−10 storage day), POV (10 and 10−4 storage days). In this study, we measured in vitro POX activity using a crude protein extract. However, in the present study, there are at least two major POX isozymes in buckwheat flour. Therefore, we may obtain clearer results if we assay the POX activity for each isozyme.

The rutin concentration had significant correlations to pH (4−0 and 10−4 storage days at 20 °C), WSA (30 storage days at 5 °C and 4 storage days at 20 °C), and COV (30 storage days at 5 °C). In addition, the rutin concentration had negative correlations to WSA at both 5 and 20 °C during the entire storage period. This result suggests that rutin inhibits fatty acid generation. To reinforce this idea, we investigated inhibitory effects of rutin against in vitro LIP activity. As a result, LIP activity was inhibited by rutin (**Figure 3**). An inhibitory efficiency of rutin against LIP activity was high at relatively lower rutin concentrations (such as 0.16 and 8 μg/mL) than at higher.

Buckwheat flour contains both LIP activity and rutin. Buckwheat flour contains rutin around 20 mg/100 g of flour. To calculate quantitatively, LIP activity in buckwheat flour can be inhibited by rutin in buckwheat flour for 40%. These results indicate that to breed a buckwheat variety that does not deteriorate easily, increasing the rutin concentration in buckwheat seed would be effective.

These results suggest that LIP activity plays important roles in the quality deterioration of buckwheat flour that relates to lipid degradation. This indicates that the mechanism of quality deterioration in buckwheat flour is different from that of rice and soybean. To breed a buckwheat variety that does not deteriorate easily, decreasing the LIP activity in buckwheat seed would be effective. To further clarify the effects of enzymes on quality deterioration, flour color, volatile compounds, and sensory analysis (organoleptic evaluation) of flavors will be investigated.

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