Characterization of Peroxidase in Tartary Buckwheat Seed

Tatsuro SUZUKI^{1*}, Dong-Hoon SHIN², Sun-Hee WOO², Yuji MUKASA³, Toshikazu MORISHITA¹, Takahiro NODA¹, Shigenobu TAKIGAWA¹, Naoto HASHIMOTO⁴, Hiroaki YAMAUCHI³ and Chie MATSUURA-ENDO¹

¹ NARO Hokkaido Agricultural Research Center, Shinsei, Memuro, Kasai-Gun, Hokkaido 082-0081, Japan

² Lab. of Plant Breeding & Plant Functional Proteomics, Dept of Crop Science, College of Agricultural, Life & Environmental Sciences, Chungbuk National University, 410, Seongbong-ro, Gaeshin-dong, Cheongju City, 361-763 Chungbuk, Korea

³ NARO Hokkaido Agricultural Research Center, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-8555, Japan

⁴ NARO Kyushu Okinawa Agricultural Research Center, 2421 Suya, Koshi, Kumamoto 861-1192, Japan

Received January 22, 2011; Accepted April 9, 2012

Tartary buckwheat is targeted as a healthy food because its food contains large amounts of polyphenols such as rutin and quercetin, especially. Quercetin is a substrate for POX activity, which sometimes plays important roles for quality changes in foods. Therefore, to obtain a basic understanding of the roles of Tartary buckwheat POX, we purified and characterized POX in Tartary buckwheat seeds. POX was purified (76.7 fold, final yield 4.77%). Tartary buckwheat seeds bore a single major POX (MW 46.8 kDa). Kinetic studies revealed that POX had lower K_m values for quercetin (0.32 mM) and *o*-dianisidine (0.316 mM) than for ABTS (1.86 mM) and guaiacol (0.958 mM). POX showed greater activity under acidic pH conditions. Optimum pH of POX was the same pH as Tartary buckwheat dough. In addition, optimum temperature was around 20°C, corresponding to the temperature where dough is usually handled. Therefore, POX in Tartary buckwheat may play an important role in quality changes in food.

Keywords: tartary buckwheat, peroxidase, characterization, purification, quality

Introduction

Plant peroxidase (EC 1.11.1.7; POX), widely distributed in higher plants (Van *et al.*, 1982), is involved in a variety of physiological functions: cell elongation (Ahmed *et al.*, 1995), defense mechanisms (Bradley *et al.*, 1992; Kolattukudy, Mohan *et al.*, 1992) and lignification (Blee *et al.*, 2003). POX also plays an important role in food quality, particularly with regard to deterioration of color and flavor (Ashie *et al.*, 1996). Primary contributors to undesirable ‘beany’ and ‘green’ flavors in soybean (*Glycine max* Merr.), aldehydes and ketones are mainly generated through lipid peroxidation. Their presence has been linked to the activity of enzymes such as lipoxygenase (EC 1.13.11.12) and POX (Matoba *et al.*, 1975; Matoba *et al.*, 1985; Anli *et al.*, 2004). POX also plays an important role in the deterioration of flavor and taste in butterbur [*Petasites japonicas* Maxim.] (Ibaraki *et al.*, 1988; Ibaraki *et al.*, 1989).

Common buckwheat (*Fagopyrum esculentum* Moench) is

considered a healthy food. In Japan, buckwheat flour is used mainly for making noodles, and its flavor and color are important factors in its quality. Buckwheat flour’s rapid deterioration (Muramatsu *et al.*, 1986) has been linked to enzymatic activities (Kondo *et al.*, 1982; Ohinata *et al.*, 1997; Suzuki *et al.*, 2004; Suzuki *et al.*, 2005). Partially characterizing POX from *F. esculentum* seeds, Kondo (1982) suggested that POX influenced the oxidation of flavonoids in such seeds. In purifying and characterizing POX activity extracted from *F. esculentum* seeds, Suzuki *et al.* (Suzuk *et al.*, 2006) showed this activity to be associated with at least two isozymes, both capable of catalysis at temperatures approaching 0°C. They further demonstrated these enzymes to have a lower K_m for quercetin than for other phenolic compounds such as guaiacol. As with lipase, POX activity has been significantly correlated with lipid deterioration in *F. esculentum* flour during storage (Suzuki *et al.*, 2004; Suzuki *et al.*, 2006).

Tartary buckwheat (*F. tataricum* Gaertner) is also known to be a useful food because it contains about 100 times more rutin in its seed than common buckwheat (Suzuki *et al.*, 2002; Suzuki *et al.*, 2004). Tartary buckwheat seeds also

*To whom correspondence should be addressed.

E-mail: tsuzu@affrc.go.jp

contains large amount of rutinoidase activity; therefore rutin in Tartary buckwheat flour is easily hydrolyzed to functional flavonoid quercetin (Yasuda *et al.*, 1994; Suzuki *et al.*, 2002). However, its flour deteriorates easily, and, as with *F. esculentum* flour, POX may have an important role in this process. Therefore, to obtain a basic understanding of the roles of *F. tataricum* POX activity in seeds, we purified and characterized POX from *F. tataricum* seeds.

Materials and Methods

Plant materials *F. tataricum* and *F. esculentum* genetic resources/varieties were grown in an experimental field at the National Agricultural Research Center for the Hokkaido Region, Memuro, Hokkaido, Japan (latitude: 42°53', longitude: 143°03'). *F. tataricum* and *F. esculentum* seeds were sown early in June and harvested in late August in 2008. Harvested seeds were dried, threshed and stored at 4°C until used for experiments.

Assay of in vitro POX activity POX activities were assayed by modification of the procedure described by Suzuki (Suzuki, *et al.*, 2006). POX activities were determined at 22°C by measuring the initial rate of decrease in absorbance at 370 nm (quercetin) or the increase in absorbance at 430 nm (*o*-dianisidine), 415 nm (ABTS; 2,2'-azino-bis-(3-ethylthiazoline-6-sulfonate)), and 490 nm (guaiacol). The assay mixture contained 200 mM buffer (at the optimal pH for POX activity for each substrate), 3 mM H₂O₂, substrate (2 mM quercetin, 12 mM *o*-dianisidine, 10 mM ABTS, or 15 mM guaiacol) and 10 µL of enzyme solution in a total volume of 200 µL.

Purification of *F. tataricum* POX Following the removal of panicles, *F. tataricum* seeds were milled. Tartary buckwheat flour (100 g fresh weight) was homogenized with 1000 mL of buffer A (50 mM acetate-LiOH buffer, pH 5.5) for about 1 h. A crude enzyme solution was obtained by centrifugation and then precipitated by 0–80% saturation with solid (NH₄)₂SO₄. The precipitate was dissolved in buffer A and dialyzed overnight against buffer A. The dialyzed enzyme solution was applied to a CM-Sepharose column (24 × 115 mm, GE Healthcare Japan, Tokyo, Japan) equilibrated with buffer A. The POX was eluted using a linear 600 ml gradient of 50 to 400 mM LiCl in buffer A. Active fractions were collected and loaded onto a Sephacryl S-200 column (24 × 66 mm, GE Healthcare Japan, Tokyo, Japan) equilibrated with buffer A. Active fractions were collected and stored at –30°C. In each purification step, POX activity was measured using guaiacol as substrate. All of the above steps were carried out at 4°C.

Determination of optimal pH, pH stability, optimal temperature, thermal stability and *K_m* value of *F. tataricum* POX

To investigate the enzyme's optimal pH, *F. tataricum* POX activity at 22°C, with quercetin, ABTS and guaiacol respectively as substrates, was determined at different pHs: 2.0 (200 mM glycine - HCl buffer), 3.0 to 4.0 (200 mM citrate - LiOH buffer), 5.0 to 6.0 (200 mM acetate - LiOH buffer), 7.0 (200 mM imidazole - LiOH buffer), 8.0 (200 mM tris - HCl buffer), or 9.0 to 10.0 (200 mM borate - LiOH buffer). To investigate pH stability of POX, the enzyme solution was incubated for 4 h at different pHs in the above mentioned buffers, and POX activity then determined using guaiacol as a substrate after addition of 500 mM acetate - LiOH buffer (pH 5.5) to adjust the pH of the reaction buffer to 5.5. To assess thermal stability, the enzyme solution was incubated for 4 hr at temperatures ranging from 0°C to 80°C prior to an assay of POX activity at 22°C using guaiacol as substrate. The *K_m* values were determined by Lineweaver-Burk plots at different concentrations of each substrate.

Protein determinations Total soluble protein concentrations were measured using a DC protein assay kit (Bio-Rad, USA) using BSA as a standard protein.

Estimation of molecular weight of *F. tataricum* POX

Gel filtration for molecular estimation was carried out using the purified POX. Purified POX was loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The molecular weight was determined using a standard curve of elution volume vs. logMW derived from standard proteins.

Results and Discussion

Species differences of POX A comparison of seed POX activities in *F. tataricum* and *F. esculentum* genetic resources/varieties (Table 1) shows that POX activity in *F. tataricum* seeds was an order of magnitude greater than that in *F. esculentum* seeds. For *F. tataricum* seeds, POX activity with guaiacol as substrate ranged from 47.5 to 87.3 A490 min⁻¹ g⁻¹ (f.w.), but was only 0.3 to 6.1 A490 min⁻¹ g⁻¹ (f.w.) for *F. esculentum* seeds. This suggests that seed POX activity in *F. tataricum* may serve different roles than seed POX in *F. esculentum*. Therefore, we purified the *F. tataricum* seed POX-containing fraction and characterized it to obtain more information about *F. tataricum* POX.

Purification of POX *F. tataricum* POX was purified (76.7 fold, final yield 4.77%) by concentration, ion-exchange chromatography and gel-filtration (Table 2). The flow through fractions from CM-Sepharose did not contain any detectable POX activity. On the other hand, at the CM-Sepharose step, two peaks indicating POX activity were eluted, one strong and another weak, as in *F. esculentum* (Suzuki *et al.*, 2006), *F. tataricum* seed POX activity consisted of at least two isozymes. Although we found two POX peaks, we

Table 1. Varietal differences of seed POX activity.

	POX activity ¹⁾ mU/g.f.w. flour
Tartary buckwheat	
Hokkai T8	87.3 ± 8.7
Nepal 1	81.2 ± 0.9
Nepal 2	61.7 ± 2.8
Yugoslavia	57.9 ± 0.9
kuqiao (HUN)	54.1 ± 6.9
putong-Qukiao	60.9 ± 1.5
Ishisoba	51.4 ± 12.4
Amur region	49.7 ± 2.4
Sakhalin Tomari region	52.3 ± 4.5
Latobia	47.5 ± 6.3
common buckwheat	
Kitawasesoba	4.0 ± 0.1
Kitayuki	3.8 ± 0.2
Kitanomashu	3.8 ± 0.5
Kanto 1	6.1 ± 0.1
Shirataki-Nishi	4.9 ± 0.4
Sumchanka	0.6 ± 0.2
cv.monteneuf	1.0 ± 0.0
HC10	2.4 ± 0.2
Tanno-Hiushinai	3.7 ± 0.4
mh-1	0.7 ± 0.2
Gan-Chao	0.3 ± 0.1

1) Changes in absorbance at 490 nm / min × 10³ enzyme = 1 unit
 Guaiacol was used as a substrate
 Data are means ± S.D. (n = 3)

purified only the one with the strong POX activity, as the other activity peak was too weak to purify. The molecular weight of *F. tataricum* seed POX from the major peak was estimated at 46.8 ± 1.1 kDa (n = 3), similar to that of POXI (46.1 kDa), a POX isozyme showing trace activity in *F. esculentum* seeds (Suzuki *et al.*, 2006). These molecular weights were also similar to those reported for POX in scented geranium (*Pelargonium graveolens*; Seok *et al.*, 2001) and oil palm (*Elaies guineensis*; Sakharov *et al.*, 2000). On the other hand, the molecular weight of POXII, the major POX isozyme in *F. esculentum* seed, is 58.1 kDa. These data indicate that the molecular weight of the major active POX fraction in *F. esculentum* and *F. tataricum* are quite different, suggesting that their characteristics may differ significantly.

Kinetic constants of POX The determination of *F.*

tataricum POX K_m values for various substrates (data not shown), showed its K_m for guaiacol (0.958 mM) to be far lower than that of isoperoxidase PC3 from *P. graveolens* (7.3 mM; Seok *et al.*, 2001) or that of a neutral peroxidase isozyme from *Brassica napus* L. (3.7 mM; Duarte-Vazquez *et al.*, 2001). *F. tataricum* POX's K_m value for ABTS (1.86 mM) was higher than that of a neutral peroxidase isozyme from *B. napus* (0.7 mM; Duarte-Vazquez *et al.*, 2001), while for *o*-dianisidine (0.316 mM) it was similar to the K_m of isoperoxidase PC3 from *P. graveolens* (0.31 mM; Seok *et al.*, 2001). Among the substrates tested, *F. tataricum* POX showed its lowest K_m value for quercetin and *o*-dianisidine. Quercetin is one of the major phenolic compounds in *F. tataricum* seeds. Given that several reports have shown that POX, along with phenolic compounds such as quercetin, play an important role in enzymatic browning (Kondo *et al.*, 1982; Francisco *et al.*, 2001), *F. tataricum* POX may be implicated in color change of buckwheat noodles. While for every substrate tested *F. tataricum* POX's K_m values were higher than corresponding ones for *F. esculentum* POX, the greater quantity of POX activity in *F. tataricum* may balance the lesser specificity of its POX.

Optimal pH, pH stability, optimum temperature and thermal stability of POX The optimum pH of *F. tataricum* POX was investigated using quercetin, ABTS and guaiacol as substrates (Table 3). For quercetin and guaiacol, the optimum pH was 6.0, though high activity was observed at pH 4.0, 5.0 and 6.0. Although *F. tataricum* POX was active on guaiacol over a pH range of 2.0 to 10.0, for quercetin its activity did not extend above pH 7.0.

The optimum pH for ABTS was pH 2.0, and activity gradually decreased with an increase of pH. Comparatively, the pH optima for *F. esculentum* POX activity are pH 8.0 for POXI, and pH 4.5 for POXII with quercetin as substrate. In addition, the pH optima for *F. esculentum* POX activity are pH 3.0 for POXII, and undetectable for POXI with ABTS as substrate (Suzuki *et al.*, 2006). This indicates that *F. tataricum* seed POX had a similar pH optima as *F. esculentum* seed POXII, which is a major POX isozyme in this species.

Table 2. Purification of POX from Tartary buckwheat seed.

Purification step	Total protein (mg)	Total activity units ¹⁾	Specific activity units / mg protein	Yield (%)	Fold
crude extract	643	376	0.585	100	1.00
concentration	161	546	3.39	145	5.79
CM-Sepharose	3.85	41.3	10.7	11.0	18.3
Sephacryl S-200	0.400	17.9	44.8	4.77	76.7

¹⁾ Changes in absorbance at 490 nm / min × 10³ enzyme = 1 unit
 POX activity was measured using guaiacol as a substrate.

Table 3. Optimum pH and pH stability of POX.

pH	optimum pH ¹⁾			pH stability ¹⁾
	quercetin	guaiacol	ABTS	guaiacol
2.0	33 ± 1.7 a	9 ± 3.3 a	100 ± 5.7 a	N.D.
3.0	68 ± 5.6 b	21 ± 7.9 ab	67 ± 1.1 b	1 ± 0.0 a
4.0	89 ± 4.6 c	68 ± 14.6 c	84 ± 3.3 c	38 ± 1.2 b
5.0	100 ± 8.2 c	93 ± 12.0 d	26 ± 2.7 d	59 ± 3.3 c
6.0	4 ± 2.7 d	100 ± 19.3 d	6 ± 1.4 e	100 ± 11.5 d
7.0	N.D.	55 ± 8.3 c	N.D.	34 ± 1.7 be
8.0	N.D.	43 ± 8.4 bc	N.D.	30 ± 1.6 be
9.0	N.D.	15 ± 2.2 ab	N.D.	23 ± 1.0 e
10.0	N.D.	21 ± 3.9 ab	N.D.	6 ± 0.7 a

¹⁾ Guaiacol and quercetin; pH 6.0 = 100, ABTS; pH 2.0 = 100

Data are means ± S.D. (n = 3), N.D.; not detectable

Different alphabets within column indicate significant difference between means using Ryan's test at p = 0.05

The pH stability of *F. tataricum* POX was greatest at pH 6.0, and diminished at increasing and decreasing pH values. This indicates that to purify *F. tataricum* seed POX, pH 5.5 is appropriate in terms of enzyme stability. With guaiacol as substrate, *F. tataricum* seed POX showed activity between 0°C and 50°C, but none above 60°C (Table 4). While its optimal temperature was 20°C, its activity at 0°C and 10°C was roughly halved. These characteristics were similar to those of POXI in *F. esculentum* seed. However *F. tataricum* POX was more stable at high temperatures than *F. esculentum* POXI or POXII. *F. tataricum* POX was stable from 0°C to 40°C, and retained activity when kept at 60°C for 4 h (Table 4). On the other hand, *F. esculentum* POXI and POXII were unstable above 40°C and quickly lost activity at 60°C (Suzuki *et al.*, 2006).

F. tataricum seed POX clearly has unique characteristics compared to *F. esculentum* POXs, as well as those of other plant species. In *F. esculentum* seeds, most of the quercetin,

rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside) are localized in the embryo (Suzuki *et al.*, 2002). The same is the case in *F. tataricum* seeds, where the majority of POX activity is also localized in the embryo. Quercetin, generated from rutin by hydrolysis (Suzuki *et al.*, 2002; Yasuda, *et al.*, 1994), serves as a substrate for peroxidase-dependent oxidation of quercetin to the anti-fungal agent 3,4-dihydroxybenzoic acid (Takahama *et al.*, 2000). Based on our results, we propose that when quercetin is used as a substrate, POX activity in *F. tataricum* seeds or flour may have a role in the generation of an anti-fungal agent along with antioxidant activity. To clarify the role of POX in *F. tataricum* seeds, further studies should investigate if there exists a clear relationship between the quality of *F. tataricum* products and the seeds' POX activity.

Acknowledgment We thank Mr. K. Abe, T. Takakura, and T. Hirao for their assistance in the field. We also thank Ms. K. Fujii and Ms. M. Hayashida for their technical assistance in the laboratory experiments. This study was supported by the NIAS Genebank Project of the National Institute of Agrobiological Sciences.

Table 4. Optimum temperature and thermal stability of POX.

°C	optimum temperature ¹⁾	thermal stability ²⁾
0	40 ± 1.5 ab	93 ± 1.1 a
10	57 ± 2.3 b	100 ± 2.9 a
20	100 ± 9.7 c	98 ± 3.6 a
30	48 ± 8.1 ab	95 ± 4.2 a
40	32 ± 2.5 a	89 ± 6.5 a
50	11 ± 0.7 d	75 ± 3.8 b
60	N.D.	46 ± 4.3 c
70	N.D.	N.D.
80	N.D.	N.D.

¹⁾ 20°C = 100, ²⁾ 10°C = 100

Data are means ± S.D. (n = 3), N.D.; not detectable

Guaiacol is used as a substrate

Different alphabets within column indicate significant difference between means using Ryan's test at p = 0.05

References

- Anli, D., and Tilak, R.M. (2004). Off-flavour development in soybeans: comparative role of some antioxidants and related enzymes. *J. Sci. Food Agric.*, **84**, 547-550.
- Ahmed, N., Chaplin, M., Trevan, M., Dey, P.M. and Brownleader, M.D. (1995). Purification and partial characterization of 'extensin peroxidase'. *Biochem. Soc. Trans.*, **23**, 154.
- Ashie, N.A., Simpson, B.K. and Smith, J.P. (1996). Mechanisms for controlling enzymatic reactions in foods. *Crit. Rev. Food Sci. Nutr.*, **36**, 1-30.
- Blee, K.A., Choi, J.W., O'Connell, A.P., Schuch, W., Lewis, N.G. and Bolwell, G.P. (2003). A lignin-specific peroxidase in tobacco

- whose antisense suppression leads to vascular tissue modification. *Phytochem.*, **64**, 163-176.
- Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*, **70**, 21-30.
- Duarte-Vazquez, M.A., Garacia-Almendarez, B.E., Regalado, C. and Whitaker, J.R. (2001). Purification and properties of a neutral peroxidase isozyme from turnip (*Brassica napus* L. var. purple Top White Globe) root. *J. Agric. Food Chem.*, **49**, 4450-4456.
- Francisco, A.T. and Juan, C.E. (2001). Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.*, **81**, 853-876.
- Ibaraki, T., Hirano, T., Yamashita, N. and Baba, N. (1988). Studies on peroxidase activity of butterbur. *Bull. Fukuoka. Agric. Res. Cent.*, **B-8**, 67-72 (in Japanese).
- Ibaraki, T. and Hirano, T. (1989). Control of peroxidase and polyphenoloxidase activities, and the preserve of chlorophylls in processing of waterboiled butterbur. *Bull. Fukuoka. Agric. Res. Cent.*, **B-9**, 85-90 (in Japanese).
- Kolattukudy, P.E., Mohan, R., Bajar, A.A. and Scherf, B.A. (1992). Plant peroxidase gene expression and function. *Biochem. Soc. Trans.*, **20**, 333-337.
- Kondo, K., Kurogouti, T. and Matubashi, T. (1982). (English title not available). *Report in Research Institute of Food Technology in Nagano Prefecture*, **10**, 150 (in Japanese).
- Matoba, T., Hidaka, H., Kitamura, K., Kaizuma, N. and Kito, M. (1975). Contribution of hydroperoxide lyase activity to n-hexanal formation in soybean. *J. Agric. Food Chem.*, **23**, 136-141.
- Matoba, T., Hidaka, H., Narita, H., Kitamura, K., Kaizuma, N., and Kito, M. (1985). Lipxygenase-2 isozymes is responsible for generation of n-hexanal in soybean homogenates. *J. Agric. Food Chem.*, **33**, 852-855.
- Muramatsu, N., Ohinata, H., Obara, T. and Matsubashi, T. (1986). (English title not available) *Food Packaging*, **30**, 94 (in Japanese).
- Ohinata, H., Karasawa, H., Muramatsu, N. and Ohike, T. (1997). Properties of buckwheat lipase and depression of free fatty acid accumulation during storage. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **44**(8), 590-593 (in Japanese).
- Sakharov, I.Y., Castillo, J., Areza, J.C. and Calae, I. (2000). Purification and stability of peroxidase of African oil palm *Elaeis guineensis*. *Bioseparation*, **9**, 152-132.
- Seok, H.L., Eun, S.K. and Mi, Y.L. (2001). Purification and characterization of a cationic isoperoxidase from scented-geranium. *Phytochem.*, **58**, 859-864.
- Suzuki, T., Honda, Y., Funatsuki, W. and Nakatsuka, K. (2002). Purification and characterization of flavonol 3-glucosidase, and its activity during ripening in tartary buckwheat seeds. *Plant Sci.*, **163**, 417-423.
- Suzuki, T., Honda, Y., Funatsuki, W. and Nakatsuka, K. (2004). In-gel detection and study of the role of flavonol 3-glucosidase in the bitter taste generation in tartary buckwheat. *J. Sci. Food Agric.*, **84**, 1691-1694.
- Suzuki, T., Honda, Y. and Mukasa, Y. (2004). Purification and characterization of lipase in buckwheat seed. *J. Agric. Food Chem.*, **52**, 7407-7411.
- Suzuki, T., Honda, Y., Mukasa, Y., and Kim, S-J. (2005). Effects of lipase, lipxygenase, peroxidase, and rutin on quality deteriorations in buckwheat flour. *J. Agric. Food Chem.*, **53**, 8400-8405.
- Suzuki, T., Honda, Y., Mukasa, Y. and Kim, S-J. (2006). Characterization of peroxidase in buckwheat seed. *Phytochem.*, **67**, 219-224.
- Takahama, U. and Hirota, S. (2000). Deglucosidase of quercetin glucosides to the aglycone and formation of antifungal agents by peroxidase-dependent oxidation of quercetin on browning of onion scales. *Plant Cell Physiol.*, **41**, 1021-1029.
- Tohyama, R., Sekizawa, N., Murai, K. and Ishiya, T. (1982). Quality change in packaged buckwheat during storage. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **29**, 501 (in Japanese).
- Van Huystee, R.B., and Cairns, W.L. (1982). Progress and prospects in the use of peroxidase to study cell development. *Phytochem.*, **21**, 1843-1847.
- Yasuda, T. and Nakagawa, H. (1994). Purification and characterization of rutin-degrading enzymes in tartary buckwheat seeds. *Phytochem.*, **37**, 133-136.