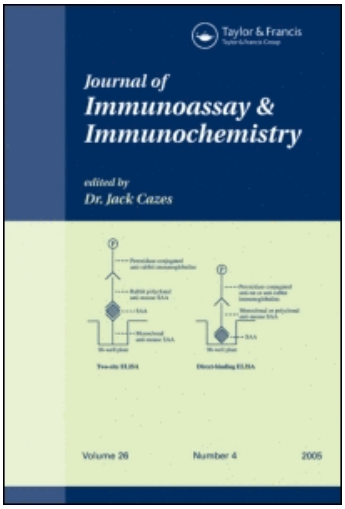

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Isolation and Some Characteristics of Lipoxygenase from Aromatic Brown Rice (*Oryza Sativa* L.) cv. Khao Dawk Mali 105

Kanya Ratchatachaiyos[a]; Chockchai Theerakulkait[b]
[a] Food Science and Technology Programme, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Phetchaburi, Thailand [b] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand

To cite this Article Ratchatachaiyos, Kanya and Theerakulkait, Chockchai(2009) 'Isolation and Some Characteristics of Lipoxygenase from Aromatic Brown Rice (*Oryza Sativa* L.) cv. Khao Dawk Mali 105', Journal of Immunoassay and Immunochemistry, 30: 1, 106 — 117
To link to this Article: DOI: 10.1080/15321810802570285
URL: http://dx.doi.org/10.1080/15321810802570285

Journal of Immunoassay and Immunochemistry, 30: 106–117, 2009

ISSN: 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810802570285

Isolation and Some Characteristics of Lipoxygenase from Aromatic Brown Rice (*Oryza Sativa* L.) cv. Khao Dawk Mali 105

Kanya Ratchatachaiyos[1] and Chockchai Theerakulkait[2]
[1]Food Science and Technology Programme, Faculty of Agricultural Technology, Phetchaburi Rajabhat University, Phetchaburi, Thailand
[2]Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand

Abstract: Lipoxygenase from Khao Dawk Mali 105 aromatic brown rice was isolated by extracting brown rice liquid nitrogen powder using 0.2 M phosphate buffer pH 7, fractionating with 30–60% $(NH_4)_2SO_4$, dialyzing and gel filtration on Sephadex G-200. The optimum pHs for dialyzed lipoxygenase activity were around 7.5 and 9.5. The enzyme appeared to be completely inactivated after heating 30 min at 70°C, 20 min at 80°C and 10 min at 90°C. The enzyme could be inhibited by $MgCl_2$, $ZnCl_2$, KCl, BHA, vitamin E, vitamin C, and BHT; however, $CaCl_2$ acted as the enzyme activator.

Keywords: Aromatic brown rice, Characteristics, Inhibition, Isolation, Khao Dawk Mali 105, Lipoxygenase

INTRODUCTION

Khao Dawk Mali 105, commonly known in food markets as "Jasmine Rice", is the most popular aromatic rice in Southeast Asia. Because of its famous reputation in appearance, cooking quality, and high aroma level, the rice has gained an increasing popularity throughout the world

Address correspondence to Chockchai Theerakulkait, Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand. E-mail: fagicct@ku.ac.th

food market.[1,2] Lipoxygenase (Linoleate oxygen oxidoreductase; EC 1.13.11.12) is a wide spread enzyme in higher plant species.[3] Lipoxygenase is a class of non-heme, iron containing dioxygenase that catalyzes the oxidation of fatty acid containing a 1,4-pentadiene system, such as linoleic acid, into the conjugated fatty acid hydroperoxide.[4] Lipoxygenase has been suggested as the cause of negative implications for the color and off-flavor of plant-based foods and also off-flavor development in various plants.[5–8] Off-flavor development, or decrease in aroma in aromatic rice grain during storage, is a serious problem that reduces the value and quality of aromatic rice. Many researchers have demonstrated that free fatty acid content, especially unsaturated fatty acid, in rice grain increases during storage.

Polyunsaturated fatty acids could be catalyzed by lipoxygenase into hydroperoxides which are further transformed into volatile compounds.[4] Suzuki et al.[9] suggested that the absence of lipoxygenase enzyme in rice grains reduces oxidative deterioration. Sanchez and Raymundo[10] also showed that off-flavor formations in aromatic rice with higher lipoxygenase activity are more than those of lower lipoxygenase activity. However, characteristics of lipoxygenase in Khao Dawk Mali 105 aromatic brown rice have not been previously investigated. Therefore, the objectives of this research were to isolate and investigate some characteristics of lipoxygenase from Khao Dawk Mali 105 aromatic brown rice.

EXPERIMENTAL

Materials

Khao Dawk Mali 105 aromatic brown rice (*Oryza sativa* L.) was vacuum packed in nylon bags and stored at −20°C until use. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO. USA: linoleic acid, polyoxoethylene-sorbitan monolaureate, ammonium sulfate, gel filtration medium Sephadex G-200, and bovine serum albumin. All other chemicals were reagent grade. Deionized distilled water was used in all experiments.

Preparation of Brown Rice Liquid Nitrogen Powder

The aromatic brown rice was frozen and blended in liquid nitrogen. The brown rice liquid nitrogen powder obtained was stored at −20°C until used for further isolation.

Extraction of Lipoxygenase

Brown rice liquid nitrogen powder was extracted with 0.2 M phosphate buffer, pH 7 (4°C) at a ratio of 2:3 (w/v) using a magnetic stirrer at 4°C for 30 min. The extracted enzyme was centrifuged at 10,000 × g 4°C for 45 min. The lipoxygenase activity in the supernatant was determined spectrophotometrically by monitoring the formation of conjugated dienes at 234 nm as described below.

Ammonium Sulfate Fractionation and Dialysis

The enzyme supernatant was 30% saturated with ammonium sulfate with continuous stirring at 4°C for 1 h. After centrifugation at 17,000 × g (4°C) for 45 min, the supernatant was 60% saturated with ammonium sulfate with continuous stirring at 4°C for 1 h. The resulting precipitate after centrifugation was dissolved in a minimum volume of 0.05 mM phosphate buffer pH 7.0 (4°C) and dialyzed using a Spectra/Por membrane (molecular weight cut off of 10,000 Daltons) against 2 L of 0.05 M phosphate buffer pH 7.0 overnight at 4°C with one change of buffer. The dialyzed solution was centrifuged at 17,000 × g (4°C) for 1 h. The supernatant of isolated lipoxygenase was stored at −20°C prior to use for investigation of some properties of the enzyme. The activity of lipoxygenase and protein content were determined at each step as described below.

Gel Filtration on Sephadex G-200

The dialyzed enzyme solution was applied to the top of a Sephadex G-200 column (1.5 × 90 cm) which was previously equilibrated with 50 mM phosphate buffer pH 7.0. Fractions of 3 mL were collected from the column with the same buffer, at a flow rate of 11 mL/h. Lipoxygenase activity and protein content in each fraction were also determined.

pH Optimum for Activity and pH Stability of Aromatic Brown Rice Lipoxygenase

To determine the pH optimum for the activity of brown rice lipoxygenase, the activity was determined spectrophotometrically in the range of pH 5.0 to 10.0. The buffer systems were 0.2 M acetate buffer pH 5.0–5.5; 0.2 M phosphate buffer pH 6.0–7.5; 0.2 M boric-borax buffer pH 8.0–9.0 and 0.2 M borax-sodium hydroxide buffer pH 9.5–10.0. In the pH stability study, the enzyme was diluted 1:10 (v/v) with the buffers

from pH 5.0 to 10.0 and incubated in capped test tubes in a water bath (25°C) for 30 min, then assayed for lipoxygenase activity. The buffer systems used for study were those for pH activity study.

Temperature Stability of Aromatic Brown Rice Lipoxygenase

The dialyzed brown rice lipoxygenase solution was diluted 1:10 (v/v) with 0.2 M sodium phosphate buffer, pH 7.0. Aliquots of diluted enzyme sample were placed in 13 × 100 mm capped test tube and incubated in a water bath at 70, 80, and 90°C for 0, 1, 2, 3, 4, 5, 10, and 20 min. Each tube was removed at a specific time, immediately cooled in ice water, and assayed for lipoxygenase activity.

Effect of Activators and Inhibitors on Aromatic Brown Rice Lipoxygenase Activity

The effects of KCl, $ZnCl_2$, $MgCl_2$, $CaCl_2$, BHT, BHA, vitamin E, and vitamin C on brown rice lipoxygenase activity were studied. The effect of each chemical solution at 200, 400, 600, and 800 μg/mL on lipoxygenase activity was determined by adding 0.1 mL of each chemical solution to 0.9 mL of enzyme solution and 2 mL of substrate solution in a cuvette. Absorbance at 234 nm (25°C) of conjugated diene formation was monitored. Percent lipoxygenase inhibition and activation was calculated by comparing units of lipoxygenase activity in the presence of activators/inhibitors to the absence of activators/inhibitors.

Lipoxygenase Activity Assay and Protein Determination

Lipoxygenase activity was determined spectrophotometrically by monitoring the formation of conjugated dienes at 234 nm over a 3 min period by a modification of the procedure described by Theerakulkait and Barret.[11] One unit of enzyme activity was defined as an increase in absorbance of 0.001 (at 234 nm)/min at pH 7.0, 25°C.

Protein was determined by the dye-binding method[12] using crystalline bovine serum albumin (BSA) as a standard. The elution profile of the protein for gel filtration column chromatography was monitored by measuring absorbance at 280 nm.

RESULTS AND DISCUSSION

The aromatic brown rice was prepared as liquid nitrogen powder for lipoxygenase isolation. The lipoxygenase was extracted from the liquid

nitrogen powder with 0.2 M sodium phosphate buffer, pH 7.0. The extraction yielded lipoxygenase activity of 13.3 units/gram of brown rice powder. Lipoxygenase was fractionated with 30–60% saturated ammonium sulfate that recovered 72.41% of total activity in the extract with 1.96-fold of purification (Table 1). Ohta et al.[13] used 37–60% saturated ammonium sulfate to fractionate lipoxygenase from rice embryo with percent recovery and purification-fold of 57.9% and 5.8-fold, respectively. In addition, Ida et al.[14] showed that rice embryo lipoxygenase was fractionated at 25–50% saturated ammonium sulfate with a percent recovery of 70%.

After dialysis, the percent recovery and purification-fold of lipoxygenase from brown rice were 63.83% and 2.53-fold, respectively (Table 1). Theerakulkait and Barret[15] showed that lipoxygenase from sweet corn germ had a recovery of 62.1% with a degree of purification of 3.0 after dialysis.

Lipoxygenase from Khao Dawk Mali 105 aromatic brown rice was further purified by using gel filtration on Sephadex G-200 column. One major peak of lipoxygenase activity was obtained and the major peaks of proteins in the dialyzed solution of solubilized 30–60% saturated ammonium sulfate precipitate were of lower molecular weight (Fig. 1). The degree of purification and percent recovery of lipoxygenase pooled from fraction number 19 to 53 were 2.88-fold and 55.05%, respectively. If a higher degree of purification was needed, the fraction number 36 to 43 should be pooled with maximum activity at fraction number 40 (114 unit/mL); however, the percent recovery of the enzyme would be decreased. Ohta et al.[13] used Sephadex G-150 to purify lipoxygenase from rice embryo; the percent recovery was only 7.2%. However, the purification-fold was surprisingly 2,980-fold. Ida et al.[14] also used

Table 1. Isolation of lipoxygenase from aromatic brown rice powder[a]

Isolation step	Total enzyme activity (unit)	Total activity (unit/g)	Specific activity (unit/mg protein)	% recovery[b]	Purification fold[c]
Crude extract	533.31	13.33	5.60	100	1.00
30–60% $(NH_4)_2SO_4$ fractionation	386.16	9.65	11.00	72.41	1.96
Dialysis	340.39	8.51	14.20	63.83	2.53
Gel filtration	293.60	7.53	16.13	55.05	2.88

[a]Based on isolation from 40 g liquid nitrogen powder (values are means of 3 replications).
[b]Relative to lipoxygenase activity in crude extract.
[c]Based on specific activity of crude extract.

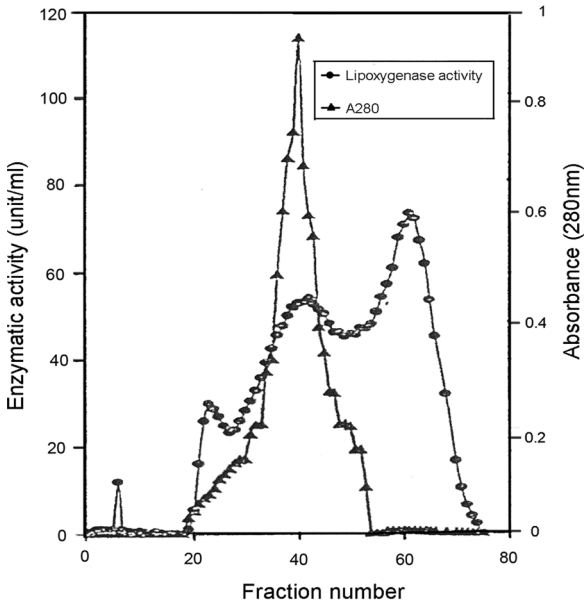

Figure 1. Sephadex G-200 Chromatographic isolation of aromatic brown rice lipoxygenase.

Sephadex G-150 to purify lipoxygenase from rice embryo and the percent recovery of lipoxygenase – 2 was only 5.4% with degree of purification of 42.3-fold.

The optimum pHs for activity of lipoxygenase from Khao Dawk Mali 105 aromatic brown rice were around pH 7.5 and 9.5 and the activity was lowest at 5.0 (Fig. 2). Kim and Rhee[16] showed that the optimum pH for activity of lipoxygenase of a milled fraction from brown rice varieties Tongjinbyeo, Kumiohbyeo, and Kanchukbyeo was in the range of pH 7.0 to 7.6. However, optimum pH activity of lipoxygenase of aromatic rice from the Philippines was pH 4.5 and 7.0.[10] Ida et al.[14] showed that the optimum pH for activity of lipoxygenase-1, lipoxygenase-2, and lipoxygenase-3 isozymes from rice (*Oryza sativa* cv. Nippon bare) embryo were 4.5, 5.5, and 7.0, respectively. Yamamoto et al.[17] showed that optimum pH for lipoxygenase activity from embryo of rice variety Japonica and Indica were around 6.5–7.0 and 8.5, respectively. The optimum pH for activity of lipoxygenase from rice bran was 6.8–7.0[18] and 8.5.[19] Moreover, Yoshie-Stark and Wasche[20] showed that the optimum pH of crude lupin lipoxygenase was around 7.5–8.0.

Lipoxygenase from Khaw Dawk Mali 105 aromatic brown rice seemed to be the most stable at pH 6.5 and 8.5 after incubation at pH 5.0–10.0, 25°C for 30 min (Fig. 3). Lipoxygenase from germinated

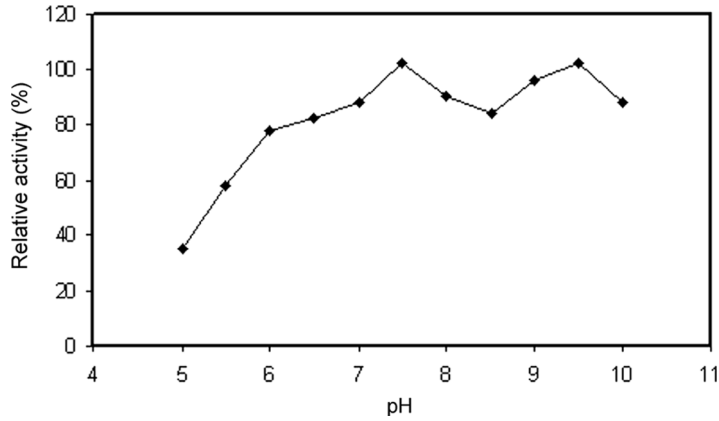

Figure 2. pH activity profile for lipoxygenase isolated from aromatic brown rice.

Japanese rice and sweet corn germ appeared to be stable at pH 5.0–8.0.[11,21] Tangwongchai et al.[22] reported that soybean lipoxygenase was stable at a pH range of 5–9. Baracat-Pereira et al.[23] also showed that lipoxygenase-1 from soybean was stable at alkaline pH, while lipoxygenase-2 was stable at neutral pH.

The temperature stability results showed that lipoxygenase activity was rapidly inactivated during the first 5 min. After heating 5 min at 70, 80, and 90°C, residual lipoxygenase activities were about 35%, 22%, and 19%, respectively. Lipoxygenase seemed to be completely inactivated after heating for 30 min at 70°C; 20 min at 80°C and 10 min at 90°C (Fig. 4). Ohta et al.[21] showed that the thermal stability of lipoxygenase-2 and

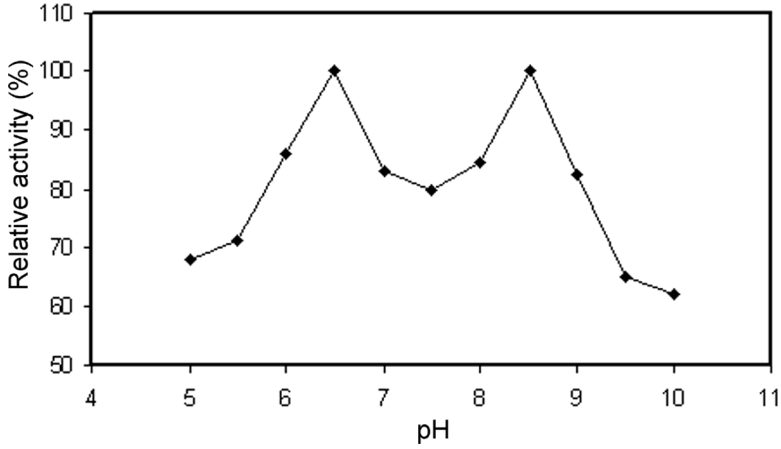

Figure 3. pH stability curve for lipoxygenase isolated from aromatic brown rice.

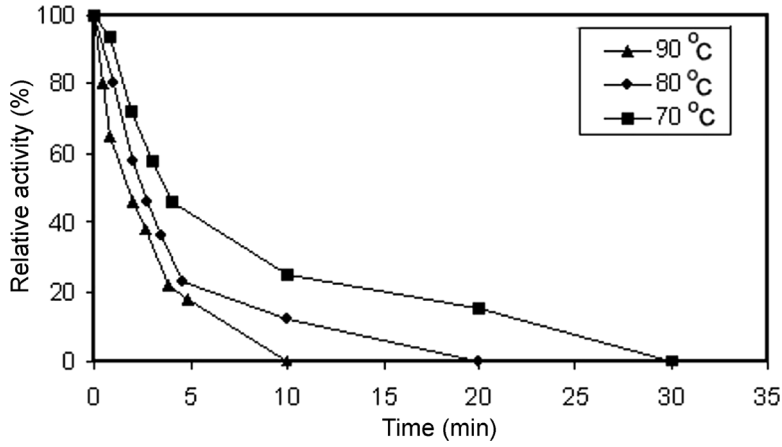

Figure 4. Temperature stability curves for lipoxygenase isolated from aromatic brown rice.

lipoxygenase-3 from rice seedings (*Oryza sativa* cv. Nippon bare) at 3 days of germination were different. The activity of lipoxygenase-3 was inactivated 10% after heating at 60°C, 20 min, while lipoxygenase-2 was inactivated only a little. Kim and Rhee[16] also showed that lipoxygenase of brown rice was inactivated by microwave heat.

Aromatic brown rice lipoxygenase was investigated for inhibition by BHA, BHT, vitamin E, and vitamin C at various concentrations. At 200 and 400 μg/mL, BHA, BHT, vitamin E, and vitamin C showed a similar inhibitory effect on brown rice lipoxygenase catalyzed conjugated diene formation. BHA inhibited conjugated diene formation more effectively than other antioxidants at concentrations of 600 μg/mL. At 800 μg/mL, BHA and vitamin E showed a similar inhibitory effect with percent inhibition of 61.77, and 58.63, respectively; however, vitamin C and BHT showed similar inhibitory effect on lipoxygenase activity (Fig. 5). Ohta et al.[21] showed that BHA and BHT inhibit activity of lipoxygenase-2 and lipoxygenase-3 from rice, but BHA showed the most inhibitory effect. Theerakulkait and Barrett[11] reported that BHA and BHT inhibited lipoxygenase activity from sweet corn germ. Lipoxygenase of cowpea was also inhibited by BHA.[24] Moreover, Boyes et al.[25] showed that BHA, BHT, and PG also inhibited activity of kiwifruit lipoxygenase. It has also been reported that peroxyl radical complexes produced from the conversion of polyunsaturated fatty acid to hydroperoxides by lipoxygenase have been reported to exist during the catalytic cycle of lipoxygenase and can serve as sources of free radicals. Therefore, antioxidants such as BHA, BHT, vitamin C, and vitamin E, which act as free radical quenchers, may act as lipoxygenase inhibitors.[26,27] The results

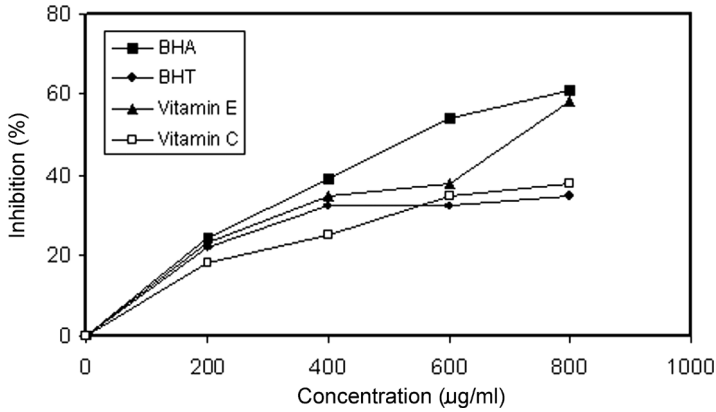

Figure 5. Effect of antioxidants on activity of lipoxygenase isolated from aromatic brown rice at various concentrations.

showed that the higher the concentration of magnesium chloride, zinc chloride, and potassium chloride, the higher percent inhibition of brown rice lipoxygenase activity. At 200, 400, and 600 µg/mL, these chlorides inhibited lipoxygenase activity to a similar extent. However, at 800 µg/mL, $ZnCl_2$ inhibited lipoxygenase activity more effectively than KCl and $MgCl_2$ with percent inhibition of 42.19, 31.31, and 29.91, respectively (Fig. 6). Hurt and Axelrod[28] also reported that magnesium ion inhibited lipoxygenase–a and –b in bush bean. Calcium chloride at 200, 400, 600, and 800 µg/mL activated the aromatic brown rice lipoxygenase activity with a percent activation of 2.88, 4.41, 14.08, and 15.81%, respectively

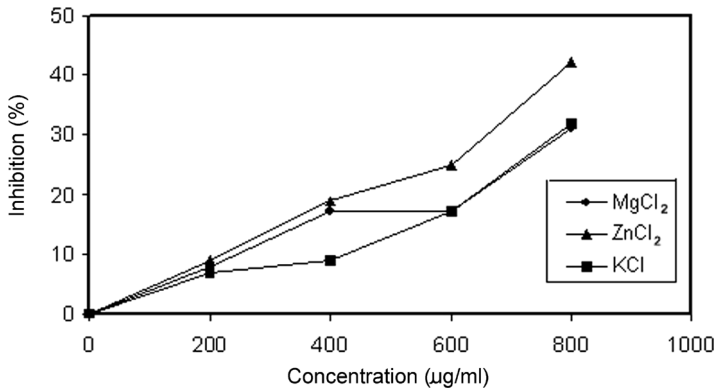

Figure 6. Effect of chloride compounds on activity of lipoxygenase isolated from aromatic brown rice.

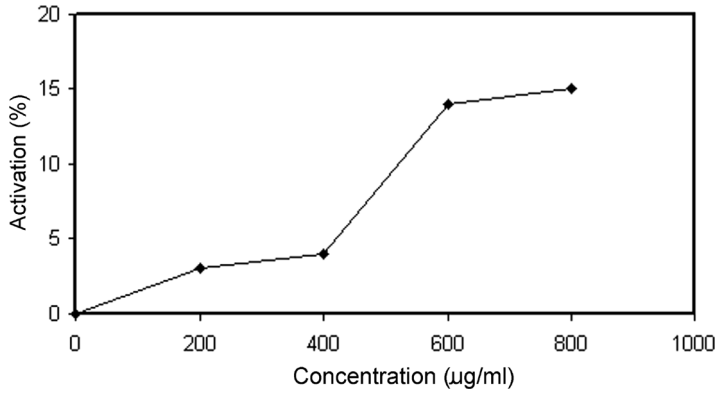

Figure 7. Effect of calcium chloride on the activity of isolated lipoxygenase from aromatic brown rice.

(Fig. 7). Activities of lipoxygenase from rice bran, peanut, and bush bean were also activated by calcium ion.[19,28,29]

ACKNOWLEDGMENTS

The authors would like to express sincere gratitude to the Kasetsart University Research and Development Institute (KURDI), Thailand for some financial support. The authors also would like to thank for Patum Rice Mill and Granary Public Co., Ltd., Thailand in kindly providing rice samples for the research. Special thanks to Dr. Dwayne Meadows for correcting the English grammar and Miss Kunnikar Boonsiripiphat for artwork preparation.

REFERENCES

1. Mahatheeranont, S.; Keawsa-ard, S.; Dumri, K. Quantification of the rice aroma compound, 2-Acetyl-1-pyrroline, in uncooked Khao Dawk Mali 105 brown rice. J. Agric. Food Chem. **2001**, *49*, 773–779.
2. Wongpornchai, S.; Dumri, K.; Jungkeawwattana, S.; Siri, B. Effect of drying methods and storage time on the aroma and milling quality of rice (*Oryza sativa* L.) cv. Khao Dawk Mali 105. Food Chem. **2004**, *87*, 407–414.
3. Grechkin, A.N. Recent developments in biochemistry of the plant lipoxygenase pathway. Progr. Lipid Res. **1998**, *37*, 317–352.
4. Babitha, M.P.; Prakash, H.S.; Shetty, H.S. Purification and properties of lipoxygenase induce in downy mildew resistant pearl millet seedling due to infection with *Sclerospora graminicola*. Plant Sci. **2004**, *166*, 31–39.

5. Barrett, D.M.; Theerakulkait, C. Quality Indicators in Blanched, Frozen, Stored Vegetables. Food Technol. **1995**, *49*, 62–65.
6. Theerakulkait, C., Barrett, D.M.; McDaniel, M.R. Sweet corn germ enzymes affect odor formation. J. Food Sci. **1995**, *60*, 1034–1040.
7. Shook, C.M.; Shellhammer, T.H.; Schwartz, S.T. Polygalacturonase, Pectinesterase, and Lipoxygenase activities in high-pressure-processed diced tomatoes. J. Agric. Food Chem. **2001**, *49*, 664–668.
8. Baysal, T.; Demirdoven, A. Lipoxygenase in fruits and vegetables: A review. Enzyme Microb. Technol. **2007**, *40*, 491–496.
9. Suzuki, Y.; Yasui, T.; Matsukura, Y.; Terao, J. Oxidative stability of bran lipids from rice variety [*Oryza sativa* (L.)] lacking lipoxygenase-3 in seeds. J. Agric. Food Chem. **1996**, *44*, 3479–3483.
10. Sanchez, T.E.; Raymundo, L.C. The lipoxygenase activity of aromatic rice. Philippine Agric. **1991**, *74*, 79–87.
11. Theerakulkait, C.; Barrett, D.M. Partial purification and characterization of sweet corn germ lipoxygenase. J. Food Biochem. **1995**, *18*, 355–372.
12. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **1976**, *76*, 248–254.
13. Ohta, H.; Ida, S.; Mikami, B.; Morita, Y. Purification and characterization of rice lipoxygenase component 3 from embryos. Agric. Biol. Chem. **1986**, *50*, 3165–3171.
14. Ida, S.; Masaki, Y.; Morita, Y. The isolation of multiple forms and product specificity of rice lipoxyganase. Agric. Biol. Chem. **1983**, *47*, 637–641.
15. Theerakulkait, C.; Barrett, D.M. Lipoxygenase in sweet corn germ: Isolation and physiochemical properties. J. Food Sci. **1995**, *60*, 1029–1033.
16. Kim, K.J.; Rhee, C.O. Lipoxygenase activity of milled fractions from brown rice. Korean. J. Food. Sci. Technol. **1997**, *29*, 145–149.
17. Yamamoto, A.; Fujii, Y.; Yasumoto, K.; Mitsuda, H. Isolation and study of some properties of rice germ lipoxygenase. Agric. Biol. Chem. **1980**, *44*, 443–445.
18. John, D.K.; Lee, G.J.; Lee, H.J. Rice bran lipoxygenase: Purification and characterization. Korean J. Biochem. **1981**, *14*, 243–252.
19. Shastry, B.S.; Raghavendra-Rao, M.R. Studies on lipoxygenase from rice bran. Cereal Chem. **1975**, *52*, 597–603.
20. Yoshie-Stark, Y.; Wasche, A. Characteristics of crude lipoxygenase from commercially de-oiled lupin for different types of lupins (*Lupinus albus, Lupinus angustifolius*). Food Chem. **2004**, *88*, 287–292.
21. Ohta, H.; Ida, S.; Mikami, B.; Morita, Y. Changes in lipoxygenase components of rice seeding during germination. Plant Cell Physiol. **1986**, *27*, 911–918.
22. Tangwongchai, R.; Ledward, D.A.; Ames, J.M. Effect of high pressure treatment on lipoxygenase activity. J. Agric. Food Chem. **2000**, *48*, 2896–2902.
23. Baracat-Pereira, M.C.; Oliveira, M.G.A.; Barros, E.G.; Moreira, M.A.; Santoro, M.M. Biochemical properties of soybean leaf lipoxygenases: Presence of soluble and membrane-bound forms. Plant Physiol. Biochem. **2001**, *39*, 91–98.

24. Den, T.V.; Mendoza, E.M.T. Purification and characterization of two lipoxygenase isoenzymes from cowpea. J. Agric. Food Chem. **1982**, *30*, 54–60.
25. Boyes, S.; Perera, C.; Young, H. Kiwifruit lipoxygenase: Preparation and characteristics. J. Food Sci. **1992**, *57*, 1390–1394.
26. Zhou, B.; Miao, A.; Yang, L.; Liu, Z.L. Antioxidative effects of flavonols and their glycosides against the free-radical-induced peroxidation of linoleic acid in solution and in micelles. Chem. Eur. J. **2005**, *11*, 680–691.
27. Banerjee, S. Inhibition of mackerel (Scomber scombrus) muscle lipoxygenase by green tea polyphenols. Food Res. Intl. **2006**, *39*, 486–491.
28. Hurt, G.B.; Axelrod, B. Characterization of two Isoenzymes of lipoxygenase from bush beans. Plant Physiol. **1977**, *59*, 695–700.
29. Nelson, M.S.; Pattee, H.E.; Singleton, J.A. Calcium activation of peanut lipoxygenase1. Lipids **1977**, *12*, 418–422.

Received June 23, 2008
Accepted July 17, 2008
Manuscript 3309