Thermal Inactivation Kinetics and Application of Phospho- and Galactolipid-Degrading Enzymes for Evaluation of Quality Changes in Frozen Vegetables

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Lipid-acyl hydrolases (LAHases) play significant roles in lipid degradation during the storage of vegetables. In particular, spinach contains a large portion of galactolipids (59.5%) and phospholipids (22.4%) among its fat-soluble components, which are used as substrates for LAHases. Thermal inactivation of various LAHases, including phospholipases A, C, and D, phosphatase, and galactolipase, from spinach and carrot was investigated to optimize the blanching process prior to the frozen storage of vegetables. Thermostability of phospholipase C or galactolipase was greatest among the LAHases from both spinach and carrot. Galactolipase from spinach exhibited a *D* value of 3.39×10^2 s at 80 °C and a *z* value of 8.21 °C, whereas phospholipase C from spinach showed D_{80} of 1.72×10^2 s with a *z* value of 9.26 °C. In the case of LAHases from carrot, the D_{65} and *z* values of galactolipase were 6.66×10^2 s and 8.69 °C, respectively, whereas phospholipase C displayed D_{85} of 3.12×10^2 s and a *z* value of 15.8 °C. Highly active and thermostable galactolipase and phospholipase C in spinach and carrot made it possible for them to be used as indicator enzymes for the determination of quality deterioration of the stored vegetables.

Keywords: *Blanching; lipid-acyl hydrolases; phospholipase C; galactolipase; thermal inactivation*

INTRODUCTION

Blanching is one of the most important steps in food processing for various frozen vegetables. The primary objective of blanching is to inactivate undesirable enzymes that cause unfavorable effects on the quality of frozen vegetables (*1*, *2*). To determine the adequacy of the blanching process, many enzymes have been suggested as indicators of sufficient heat treatment. Among various enzymes, peroxidase has been the most popular indicator enzyme in the blanching process. However, there are some problems associated with the use of peroxidase as an indicator of adequate blanching for vegetables (*3*, *4*). Heating vegetables to a temperature high enough to inactivate peroxidase is generally more than enough to destroy the undesirable enzymes responsible for quality loss in vegetables, but there is no evidence that peroxidase is directly related to specific quality deterioration of frozen vegetables. Hence, the use of peroxidase as an indicator of the blanching process may result in the loss of color, flavor, texture, and nutrients of vegetables.

Williams et al. (*3*) proposed to take lipoxygenase as an indicator enzyme for a routine assay to determine satisfactory blanching of green peas and green beans. Barrett and Theerakulkait (*2*) investigated the merits of using lipoxygenase as an appropriate blanching indicator instead of peroxidase. However, although many vegetables showed easily measurable lipoxygenase activities, the activity of lipoxygenase has not been detected in some plants including spinach and carrot (*3*).

In general, triglyceride-degrading lipases are not found in green plants, whereas highly active phospho- and galactolipases are widely detected (*5*, *6*). Furthermore, their enzymatic substrates such as phospho- and galactolipid are the major plant lipids as shown in Table 1 (*7*, *8*). Therefore, various enzymes degrading the phospho- and galactolipids seem to play an important role in the quality deterioration of vegetables and fruits (*9*).

In galactolipids, glycerol is acylated at the C-1 and C-2 positions and a glycosidic linkage is created at C-3 by galactose to form monogalactosyl or digalactosyl diglyceride. The associated fatty acids in glycolipids are highly unsaturated (*10*). In contrast, the most common phospholipids are phosphatidyl esters in which phosphatidic acid is esterified with another hydroxy com-

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10.1021/jf001379b CCC: $20.00
Published on Web 04/03/2001

Table 1. Lipid Contents in Spinach and Carrot

	spinach	carrot
total lipid (TL; %, dry basis)	5.02 ± 0.14	1.40 ± 0.27
neutral lipid (% in TL)	16.9 ± 1.28	9.00 ± 1.41
galactolipid (% in TL)	59.5 ± 2.90	65.9 ± 4.99
phospholipid (% in TL)	22.4 ± 1.61	25.2 ± 6.38

pound to form a diester of phosphoric acid. Incorporation of choline or ethanolamine as a hydroxy compound results in the formation of phosphatidylcholine (lecithin) and phosphatidylethanolamine (cephaline), respectively. There are enzymes that specifically hydrolyze one or more ester bonds in phospholipids and/or galactolipids (*11*, *12*).

During the storage of unblanched pea at −17.8 °C, Lee and Mattick (*13*) observed large losses of fatty acids in phospholipids. Pendlington (*14*) also reported that the content of phospholipids decreased while that of choline and phosphate increased during the storage of unblanched pears, implying the involvement of phospholipases in the degradation of phospholipids in unblanched vegetables. Hirayama and Oido (*7*) investigated changes of lipid and pigment contents in spinach leaves during storage and showed that lipid decreased more rapidly than pigment. Also, it was found that galactolipids, the main components in the chloroplast of plants, started to decompose at a higher rate at the earlier stage of storage. Although these results indicated that phospholipid- and galactolipid-degrading enzymes triggered undesirable effects on the quality maintenance of various vegetables during storage, the kinetics of heat stability of lipid-acyl hydrolases (LAHases) in plant tissues has not been determined systematically. In this study, we investigated the thermal inactivation of LAHases including phospholipase A (PLA), phospholipase C (PLC), phospholipase D (PLD), phosphatase, and galactolipase (GL) from spinach and carrot. In addition, the possibility of utilizing LAHases, such as PLC and GL, as indicator enzymes for the thermal process of vegetables was proposed.

MATERIALS AND METHODS

Samples and Chemicals. Spinach and carrots were purchased from a local market in Suwon, South Korea. Freeze-dried spinach powder was used for the experiments except for the preparation of GL, and fresh carrots were utilized for the crude enzyme preparation. Phosphatidylcholine, *p*-nitrophenyl phosphorylcholine, *p*-nitrophenol, *p*-nitrophenyl phosphate, and digalactosyl diglyceride were obtained from Sigma Chemical Co. (St. Louis, MO). Lecithin from Lucas Meyer (Hamburg, Germany) was used for the experiment. All other chemicals were of reagent grade.

Determination of Fat-Soluble Components in Spinach Leaves and Carrots. The samples, freeze-dried spinach powder (20 g) or ground fresh carrots (50 g), were mixed twice with 200 mL of chloroform/methanol (2:1, v/v) and filtered using a Büchner funnel. One-fifth volume of distilled water was added into the filtrate, and the chloroform layer was separated from the water layer. After elimination of the water layer, the solvent was removed from the chloroform layer using a rotary vacuum evaporator (Eyela N-1, Tokyo, Japan). The dried sample was considered as total lipid (TL) and stored at −20 °C under nitrogen gas for further experiments. For the fractionation of total fat-soluble materials from the TL sample, salicylic acid column chromatography (SCC) was performed. After 300 mg of TL had been dissolved into 2−3 mL of chloroform, the sample was loaded into the glass column (30 cm × 2.5 cm) packed with activated salicylic acid (200−400 mesh, Sigma Chemical Co.). Neutral lipid, galactolipid, and phospholipid were fractionated by eluting with chloroform (250 mL), acetone (250 mL), and methanol (250 mL), respectively. Each fraction was dried with a rotary vacuum evaporator and stored at −20 °C as described above. The amounts of each fat-soluble component were determined by densitometer GS-670 (Bio-Rad, Hercules, CA) after thin-layer chromatography (TLC). The samples were loaded on silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) and developed with chloroform/methanol/distillized water (65:25:4, v/v/v) for 1.5 h. After visualization by spraying H_2SO_4 (46.5%) solution, the amounts of fat-soluble components were measured by determining the density of spots using Bio-Rad densitometer GS-670. Standards for TLC analysis were monolinolein, monolinolenin, dilinolein, dilinolenin, trilinolein, trilinolenin, palmitic acid, linoleic acid, sitosterol, monogalactosyl diglyceride, digalactosyl diglyceride, sulfoquinovosyl diglyceride, phosphatidylserine, phosphatidylcholine, phosphatidylinositol, phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine, and cerebroside. All reagents were purchased from Sigma Chemical Co. except sulfoquinovosyl diglyceride, which was obtained from Larodan AB (Malmo, Sweden).

Enzyme Preparation from Spinach. Freeze-dried spinach powder (300 g) was suspended in 2.5 L of 20 mM Tris-HCl buffer (pH 7.5). The supernatant was collected by centrifugation at 5500*g* for 30 min. The crude enzyme was fractionated between the concentrations of 15% (w/v) and 65% (w/v) ammonium sulfate at 4 °C. After centrifugation at 5500*g* for 30 min, the pellet was dissolved in 200 mL of 20 mM Tris-HCl buffer (pH 7.5), and the resulting solution was dialyzed against 5 L of the same buffer. The supernatant obtained after centrifugation at 5500*g* for 30 min was used as crude enzyme solution for the assay and characterization of PLA, PLC, PLD, and phosphatase. Because of the shortage of freeze-dried spinach sample, fresh spinach was used for the preparation of GL instead. Fresh spinach (600 g) was ground and suspended in 3 L of 100 mM potassium phosphate buffer (pH 5.6) containing 0.4 M sucrose. The spinach solution was homogenized for 30 s at 19000 rpm using homogenizer T 25B (IKA, Staufen, Germany). The ammonium sulfate fractionation (65%) was performed as described above except for the use of 50 mM potassium phosphate buffer (pH 5.6) and 1 mM EDTA for the resuspension and dialysis of crude enzyme solution.

Enzyme Preparation from Carrot. Fresh carrots (1 kg) were cut into small pieces (1 × 1 cm^2) and blended with 1 L of 20 mM Tris-HCl buffer (pH 7.5). The supernatant was collected by centrifugation at 5500*g* for 30 min after filtration with cheesecloth. The crude enzyme was fractionated with 65% (w/v) ammonium sulfate and kept overnight in a cold chamber (4 °C). The precipitated proteins were collected with centrifugation, resuspended with 100 mL of 20 mM Tris-HCl buffer (pH 7.5), and dialyzed against 5 L of the same buffer. The supernatant obtained after centrifugation at 5500*g* for 30 min was used as crude enzyme solution for enzyme assays. In the case of PLA, 4 kg of fresh carrots was utilized for the preparation of enzyme solution.

Determination of Phospholipase A Activity. Forty milliliters of substrate solution containing 1% lecithin (w/v, Lucas Meyer) and 1% Triton X-100 (w/v, Sigma Chemical Co.) was mixed with 1 mL of crude enzyme solution. The reaction mixture was incubated for 10 min at 37 °C. The fatty acids produced by enzyme reaction were titrated with NaOH (0.01 M) using autotitrator DL77 (Mettler, Weighing, Switzerland) (*15*). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of fatty acids per minute at 37 °C.

Determination of Phospholipase C and D Activities. The enzyme assay for PLC was performed as described by Kurioka (*16*). Three hundred microliters of the reaction mixture containing 70 μL of 100 mM glycine−NaOH buffer (pH 9.0), 30 μL of 50 mM *p*-nitrophenyl phosphorylcholine (Sigma Chemical Co.), 150 μL of distilled water, and 50 μL of enzyme solution was incubated at 60 °C for 30 min. To stop the enzyme reaction, 400 μL of ethanol and 100 μL of 2 M Tris solution were added into the reaction mixture. The yellow color produced by *p*-nitrophenol was measured using spectrophotometer Ultrospec III (Pharmacia, Uppsala, Sweden) at 400

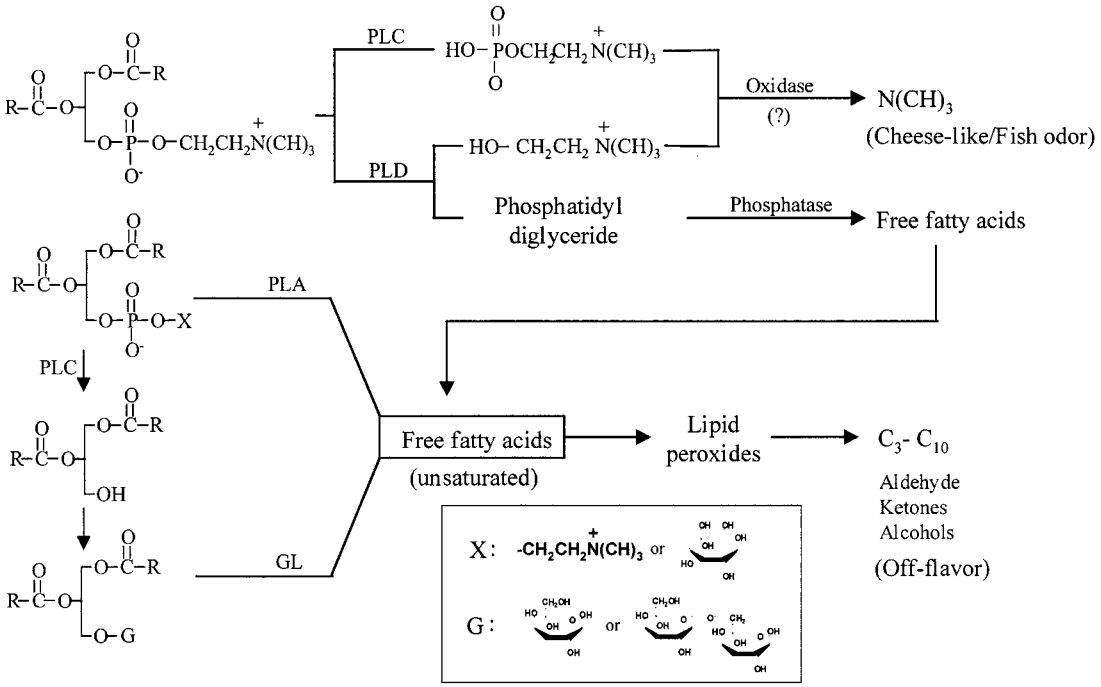

Figure 1. Possible degrading pathways of phospho- and galactolipids during storage of vegetables (*1*). PLA, phospholipase A; PLC, phospholipase C; DGC, diglyceride; GL, galactolipase; PLD, phospholipase D.

nm. The enzyme activity was calculated from a standard curve using *p*-nitrophenol as a reference. One unit of enzyme was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute at 60 °C.

Phospholipase D activity was measured by employing the method described by Abdelkarim et al. (*17*). The substrate, phosphatidylcholine emulsion, was prepared by sonicating 10% (w/v) phosphatidylcholine in distilled water for 2 min in an ice bath. The reaction mixture (100 μL) was composed of 25 μL of 10% (w/v) phosphatidylcholine emulsion, 25 μL of 20 mM sodium citrate buffer (pH 6.0), 12.5 μL of 10 mM $CaCl_2$, 37.5 μL of 2.25% (w/v) Triton X-100, and 25 μL of enzyme solution. After 30 min of incubation at 37 °C, 50 μL of 50 mM EDTA in 1 M Tris-HCl (pH 8.0) was added, and the final mixture was immediately boiled for 5 min to stop the enzyme reaction. For the spectrometric detection of the released choline, 1 mL of the color development solution (3 units of choline oxidase, 3 units of peroxidase, 5 mM 4-aminoantipyrine, and 8 mM phenol in 10 mM Tris-HCl, pH 8.0) was supplied to the reaction mixture. After incubation for 20 min at 37 °C, the absorbance was measured at 500 nm. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of choline per minute at 37 °C.

Determination of Acid Phosphatase Activity. The enzyme solution (100 μL) was mixed with the substrate containing 60 μL of *p*-nitrophenyl phosphate (50 mM) and 140 μL of 100 mM sodium citrate buffer (pH 4.0). After incubation at 37 °C for 30 min, 800 μL of ethanol and 200 μL of 2 M Tris solution were added to stop the reaction (*18*). The absorbance produced by *p*-nitrophenol was measured at 400 nm using spectrophotometer Ultrospec III (Pharmacia). One unit of acid phosphatase was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *p*-nitrophenol per minute.

Determination of Galactolipase Activity. For GL reaction, the substrate solution composed of 30 μL of digalactosyl diglyceride (0.25%, w/v), 5 μL of Triton X-100 (2.4%, w/v), and 5 μL of 100 mM potassium phosphate buffer (pH 5.6) was mixed with enzyme solution (40 μL) and incubated for 30 min at 30 °C (*19*). The enzyme reaction was stopped by incubating the reaction mixture in boiling water for 5 min. After centrifugation at 10000*g* for 10 min, the reaction products were analyzed by TLC (*1*). The supernatant was loaded on silica gel 60 F_{254} TLC plates (Merck) and developed with chloroform/methanol/distilled water (65:25:4, v/v/v) for 1.5 h. After visualization of the product by spraying H_2SO_4 (46.5%) solution, the amount of fatty acid or nonhydrolyzed digalactosyl diglycerides was measured by determining the density of spots using Bio-Rad densitometer GS-670.

Thermal Inactivation of Enzymes. The thermal inactivation experiment was performed as described by Chang et al. (*20*) with some modification. Three milliliters of buffer (20 mM Tris-HCl, pH 7.5) was transferred to a test tube with cap and stored in a water bath to equilibrate the temperature at the inactivation temperature. Then, 30 μL of an enzyme solution was added to the test tube. Temperatures and time for heat treatment were chosen according to the optimum reaction temperature of each enzyme. During heat treatment, a 0.2 mL of aliquot was taken at specific time intervals and kept in an ice bath. In case of the thermal inactivation of PLA, a 1 mL of aliquot was sampled for the determination of residual activity. The residual enzyme activities for each LAHase were measured as described above.

Estimation of Kinetic Parameters for Thermal Inactivation Reactions. The rate constant for thermal inactivation reactions of LAHases was calculated from the initial slope of the thermal inactivation curves because the enzymes were inactivated mainly during the early stage of heating, although some of the inactivation data showed biphasic kinetic curves. Other kinetic parameters were obtained by eq 1 and 2

$$2.3 \log \frac{k}{T} = 2.3 \log \frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{T} - \frac{\Delta H^{\ddagger}}{T} \quad (1)$$

where k is the rate constant of the inactivation reaction, k_B is the Boltzmann constant, h is the Planck constant, and T is an absolute temperature.

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (2)$$

RESULTS AND DISCUSSION

Galactolipid- and Phospholipid-Degrading Enzymes as Possible Indicator Enzymes in Vegetables. The fat-soluble components in spinach leaves and carrots were extracted, and various components were fractionated using SCC as described under Materials and Methods. As shown in Table 1, the total lipids

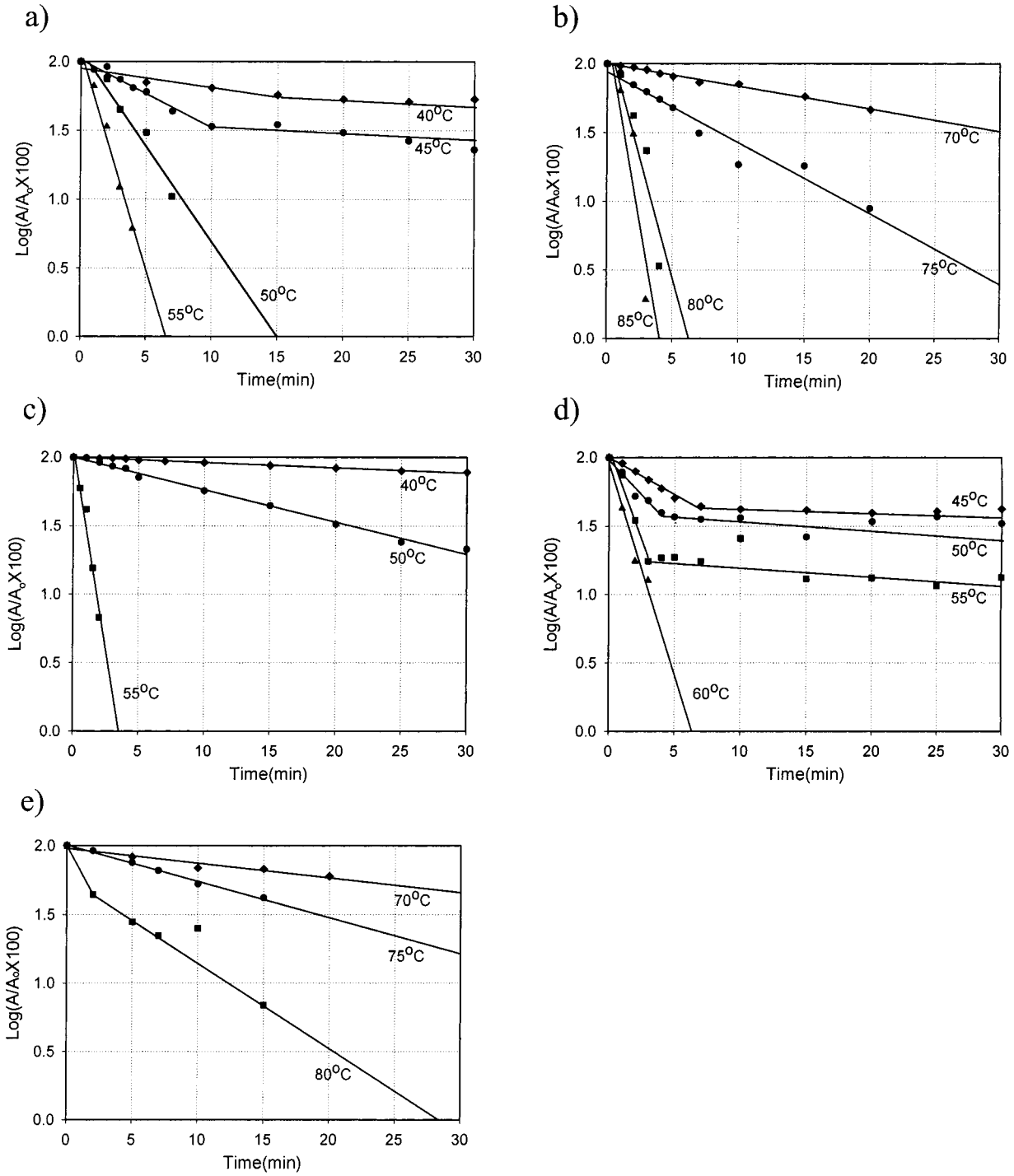

Figure 2. Thermal inactivation of PLA (a), PLC (b), PLD (c), phosphatase (d), and galactolipase (e) from spinach.

in spinach and carrot were 5.10 and 1.40%, respectively. Among total lipids, the fractions of phospho- (22.4%) and galactolipids (59.5%) were dominant compared with neutral lipid (16.9%) in spinach. These data were consistent with the result obtained by Hirayama and Oido (*7*). They observed that the contents of neutral lipid, galactolipid, and phospholipid in spinach leaves were 6, 17, and 66%, respectively. Although the portion of total lipids in carrot was lower than in spinach, the lipid compositions in both spinach and carrot were very similar (Table 1). Accordingly, it is presumed that phospho- and galactolipids are the major lipids in plants, and various enzymes possessing catalytic activities to decompose these lipids may play a pivotal role in the quality loss of many vegetables.

Many researchers have studied the mechanisms for the formation of volatiles as the results of oxidation of lipids in vegetables (*20*, *22*). The enzymes involved in this process are lipases, phospholipid-degrading enzymes, galactolipid-degrading enzymes, and lipoxygenase. Lipids are hydrolyzed to give free fatty acids, and the resulting unsaturated free fatty acids are likely to be oxidized to form lipid peroxides by the action of an enzyme, iron−sulfur center, autoxidation, or photooxygenation. In spinach leaves, unsaturated fatty acids (18:3) are present in a large portion of >73−87% (*10*). Thus, those free fatty acids could be very rapidly oxidized and forming various peroxides. These resulting peroxides are then decomposed to various aldehydes, ketones, and alcohols, causing off-flavors in food, by a

Table 2. Thermodynamic Constants for the Inactivation of Phospholipid- and Galactolipid-Degrading Enzymes

	T (°C)	K^a (1/s)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/mol·K)	$\Delta G^{\ddagger}$ (kJ/mol)
		Spinach			
PLA	40	0.84 ± 0.18		275.6	87.7
	45	3.00 ± 0.18		277.3	85.8
	50	8.28 ± 1.08	174 ± 10.3	277.1	84.5
	55	18.96 ± 1.50		275.6	83.6
PLC	70	0.96 ± 0.06		454.7	96.0
	75	3.12 ± 0.24		453.8	94.1
	80	20.94 ± 4.68	252 ± 38.0	459.2	90.0
	85	32.76 ± 10.08		452.9	90.0
PLD	40	0.24 ± 0.00		539.9	90.1
	50	1.44 ± 0.06	260 ± 99.7	528.8	89.2
	55	35.10 ± 3.66		542.9	81.9
phosphatase	45	3.24 ± 0.18		64.1	85.6
	50	6.06 ± 0.78		64.0	85.3
	55	15.60 ± 1.92	106 ± 17.9	66.7	84.1
	60	18.48 ± 2.46		63.1	85.0
GL	70	0.66 ± 0.96		521.6	97.1
	75	1.56 ± 0.06	276 ± 63.7	517.0	96.1
	80	10.62 ± 0.00		521.6	91.9
		Carrot			
PLA	45	1.86 ± 0.18		251.3	87.1
	50	3.24 ± 0.17	167 ± 44.2	247.6	87.0
	55	13.26 ± 1.56		251.3	84.6
PLC	70	1.44 ± 0.12		105.3	94.9
	75	4.32 ± 0.72		108.8	93.1
	80	5.88 ± 1.20	131 ± 20.8	105.9	93.6
	85	11.64 ± 0.48		106.3	92.9
PLD	50	3.00 ± 0.22		154.1	87.2
	55	4.56 ± 1.01		151.0	87.4
	60	11.4 ± 2.22	137 ± 17.5	152.2	86.3
	65	29.34 ± 0.00		153.8	85.0
phosphatase	55	1.86 ± 0.12		213.7	90.0
	60	4.26 ± 0.18		213.1	89.0
	65	13.68 ± 0.72	160 ± 14.8	215.6	87.1
	70	23.22 ± 1.86		212.9	87.0
GL	55	0.36 ± 0.03		462.2	94.4
	60	1.74 ± 0.24	246 ± 21.4	463.9	91.5
	65	5.40 ± 0.24		462.3	89.7

[a] Measurements were taken in duplicate.

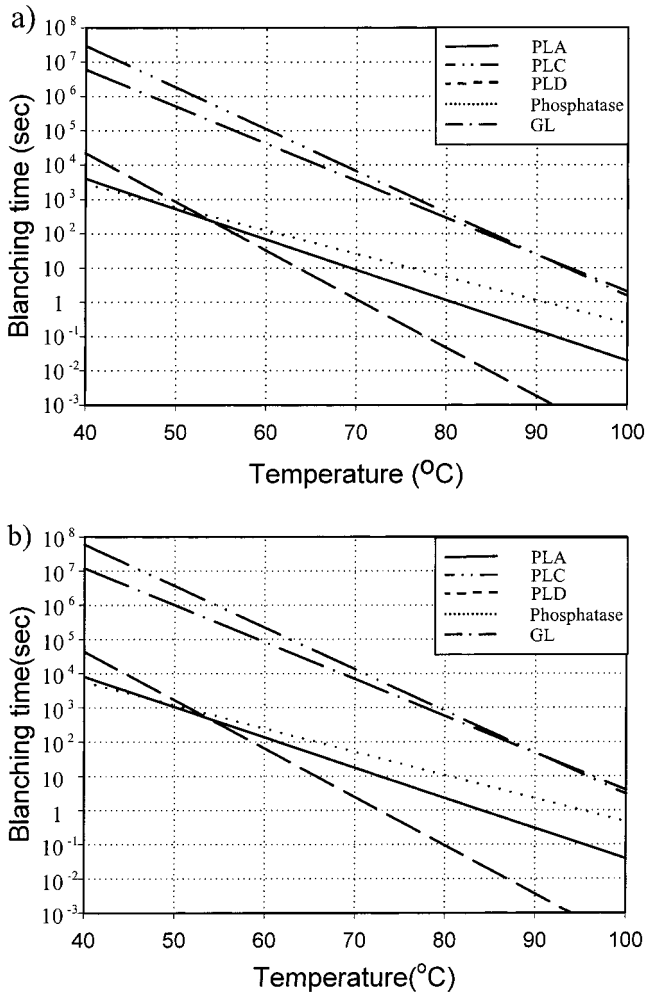

Figure 3. Nomograph for the determination of 10% (a) and 1% (b) residual activities of various LAHases from spinach.

specific enzyme or a chemical reaction. The common C_3–C_{10} aldehydes, ketones, and alcohols derived from oxidative unsaturated fatty acid breakdown are detected in many vegetables and fruits (Figure 1).

Williams et al. (*3*) examined the lipoxygenase activity of various vegetables and fruits by using a rapid KI–starch method. Although many vegetables and fruits exhibited easily detectable lipoxygenase activities, the activity of lipoxygenase has not been detected in spinach and carrot (*3*). Therefore, galactolipid- and/or phospholipid-degrading enzymes(s) may be mainly responsible for providing initial free fatty acids, which could be oxidized to form various peroxides. Free fatty acids were most likely to be produced from phospholipids via an alternative pathway. First, phosphatidylcholine would be hydrolyzed to diglyceride and phosphorylcholine, and the resulting diglyceride is converted to galactolipid in the presence of galactose in spinach leaves (*23*). The galactolipid produced would be readily hydrolyzed further to free fatty acid by GL. Hence, the phospholipid- and galactolipid-degrading enzymes might play an important role in quality deterioration of vegetables during storage, and those enzymes could be used as indicator enzymes for the blanching process.

Thermal Inactivation of Phospho- and Galactolipid-Degrading Enzymes from Spinach. To investigate the possibility of utilizing LAHases as indicator enzymes for quality maintenance of frozen vegetables, examination of the thermal characteristics of various

Table 3. *D* and *z* Values of Phospholipid- and Galactolipid-Degrading Enzymes in Spinach and Carrot

enzyme	*D* values (s)										*z* (°C)
	40 °C	45 °C	50 °C	55 °C	60 °C	65 °C	70 °C	75 °C	80 °C	85 °C	
					Spinach						
PLA	4610	1200	508	204							11.4
PLC							3650	1160	172	72	9.26
PLD	16200		2040	90							7.06
phosphatase		1440	564	213	146						14.7
GL							5600	2280	339		8.21
					Carrot						
PLA		1920	906	270							11.7
PLC							2510	828	468	312	15.8
PLD			1180	774	316	122					15.0
phosphatase				1940	660	263	155				13.5
GL				9420	2100	666					8.69

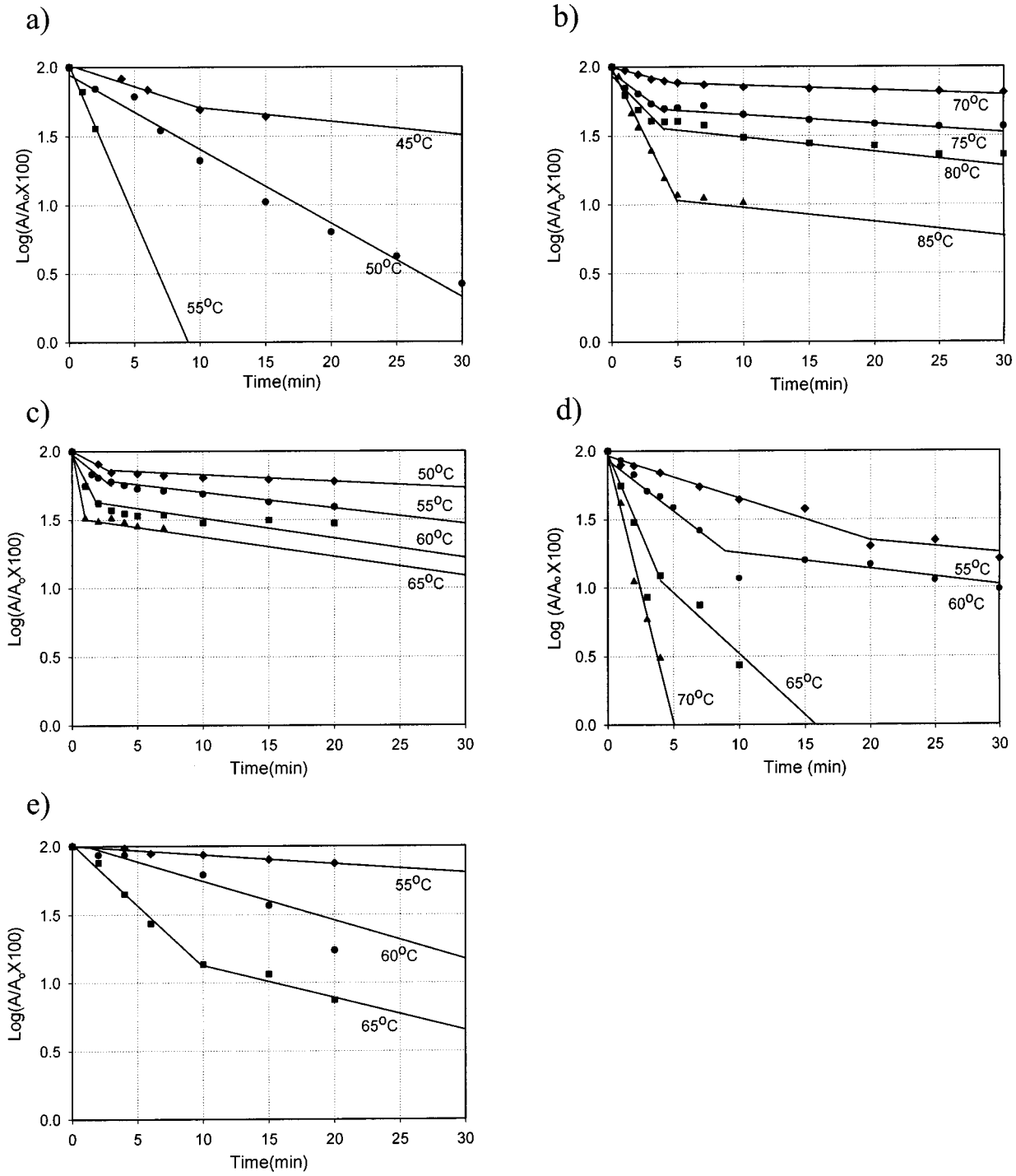

Figure 4. Thermal inactivation of PLA (a), PLC (b), PLD (c), phosphatase (d), and galactolipase (e) from carrot.

phospho- and galactolipid-degrading enzymes was prerequisite. Spinach and carrot were used as model vegetables in this experiment because the detectable lipoxygenase, the most popular indicator enzyme, has not been detected (*3*).

Thermal inactivation of PLA, PLC, PLD, phosphatase, and GL from spinach was performed at various heating temperatures, and the results are shown in Figure 2. When the thermal inactivation data of the LAHases were plotted semilogarithmically, some of them showed biphasic kinetic curves with initial steeper slopes, whereas others displayed typical linear first-order inactivation kinetic curves. Biphasic thermal inactivation kinetic curves have been reported also for peroxidase (*20*, *24*) and lipoxygenases (*25*). For the case of biphasic inactivation, the reaction rate constants were calculated by the slope of the initial steep curve and are listed in Table 2, assuming that major thermal inactivation would have occurred during the initial first-order inactivation of the process.

The purified enzymes could show significantly different stability from those present in the plant tissue because the cell components might stabilize or destabilize the enzymes. Hence, the crude cell extract was used as an enzyme solution for the determination of enzyme activities in this experiment. Larger *D* values in the enzyme inactivation data imply longer times required to inactivate 90% of the initial enzyme activity and indicate the enzyme is more thermostable than an enzyme with smaller a *D* value at a certain temperature.

The galactolipid-degrading enzyme, GL, showed the greatest thermostablity with a D value of 3.39×10^2 s at 80 °C (Table 3). In contrast, the phospholipid-degrading enzymes, for example, PLC, exhibited a lower D value at 80 °C ($D_{80} = 1.72 \times 10^2$ s) than GL. Other phospholipid-degrading enzymes including PLA, PLD, and phosphatase were even less thermostable than PLC and GL in spinach, displaying D_{55} between 0.9×10^2 and 2.13×10^2 s. The dependence of D value on the heating temperature could be expressed as z value, and this value represented the sensitivity of the enzymes to the change of heating temperature. From the z value, it was determined that phosphatase was the least sensitive to the increase of heating temperature among LAHases (z value of phosphatase = 14.7 °C), whereas PLC and GL exhibited lower z values of 9.26 and 8.21 °C, respectively.

Figure 3 shows the nomographs for various phospho- and galactolipid-degrading enzymes in spinach to retain 1 or 10% residual activities under the conditions of various temperature−time combinations. This indicated that PLC and GL required more heating time to inactivate the enzyme activity than any other enzymes in spinach. By employing the heat treatment temperature and time on the basis of this nomograph, the deterioration of quality caused by LAHases can be prevented in spinach.

Thermal Inactivation of Phospholipid- and Galactolipid-Degrading Enzymes in Carrot. Thermal inactivation of PLA, PLC, PLD, phosphatase, and GL from carrot was carried out at various heating temperatures as described under Materials and Methods. Similar to the results for spinach, biphasic thermal inactivation curves were observed in PLC, PLD, and phosphatase from carrot (Figure 4). The reaction rate constants and D values of enzymes were calculated from the initial slope because the enzymes were inactivated mainly during the early stage of heating as shown in Tables 2 and 3.

D and z values of various LAHases in carrot obtained by thermal inactivation experiments are shown in Table 3. Although the D value of PLC at 85 °C was 3.12×10^2 s, D_{65} values of other LAHases such as PLA, PLD, phosphatase, and GL were $< 6.66 \times 10^2$ s. Hence, it was considered that PLC was the most thermostable enzyme among LAHases followed by GL in carrot. In comparison with GL in spinach, D values of GL in carrot exhibited very small values, indicating GL in carrot was less thermostable than that in spinach. The z values of phospholipid-degrading enzymes were in the range of 11.7−15.8 °C, whereas that of GL (z value = 8.69 °C) was smaller. Therefore, GL is the enyzme most sensitive to the increase of heating temperature among LAHases in carrot.

Nomographs for the determination of 1 or 10% residual activities of LAHases from carrot are displayed in Figure 5. When blanching of the carrot is performed above 50 °C, PLC is the first choice of indicator enzyme among LAHases, and it requires 5.02×10^3 s to decrease PLC activity to 1% of initial enzyme activity at the heating temperature of 70 °C. Therefore, PLC can be utilized as an indicator enzyme to prevent quality losses caused by LAHases during the storage of processed carrot (Table 3).

Conclusions. It was presumed that free fatty acids produced by the enzymatic action of phospholipases and GL were oxidized readily to lipid peroxides. Off-flavors from the deteriorated vegetables resulted from the oxidation products created by the action of free fatty acids and/or peroxides degrading enzymes. Phospholipid and galactolipid (Table 1) among the fat-soluble components in spinach and carrot could result in the production of free fatty acids and further oxidized products by the action of phospholipases and GL. Therefore, phospholipases and GL might play a critical role in the quality deterioration of vegetables during storage. To prevent the quality loss of vegetables caused by LAHases and to optimize the blanching process, thermal inactivation experiments of phospholipid- and/or galactolipid-degrading enzymes in spinach and carrot were carried out. PLC from both spinach and carrot showed the greatest thermostability, and GL from spinach was also significantly thermostable. Hence, PLC and GL among LAHases could be used as suitable indicator enzymes for optimizing the blanching process of various vegetables.

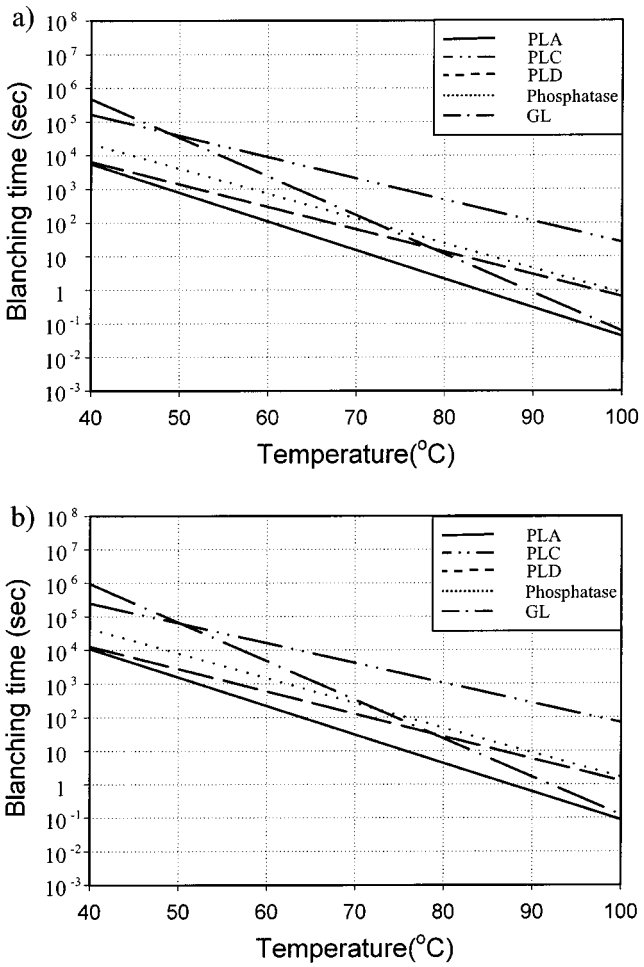

Figure 5. Nomograph for the determination of 10% (a) and 1% (b) residual activities of various LAHases from carrot.

ABBREVIATIONS USED

LAHases, lipid-acyl hydrolases; PLA, phospholipase A; PLC, phospholipase C; PLD, phospholipase D; GL, galactolipase; TL, total lipid; SCC, salicylic acid column chromatography; TLC, thin-layer chromatography.

LITERATURE CITED

(1) Park, K. H.; Kim, J. W.; Kim, M. J. Application of enzymatic reactions for evaluation of quality changes in frozen vegetables. In *Chemical Markers for Processed and Stored Foods*; Lee, T. C., Kim, H. J., Eds.; ACS Symposium Series 631; American Chemical Society: Washington, DC, 1996; pp 159−178.

(2) Barrett, D. M.; Theerakulkait, C. Quality indicators in blanched, frozen, stored vegetables. *Food Technol*. **1995**, *49*, 62−65.

(3) Williams, D. C.; Lim, M. H.; Chen, A. O.; Pangborn, R. M.; Whitaker, J. R. Blanching of vegetables for freezing-which indicator enzyme to choose. *Food Technol.* **1986**, *40*, 130−140.

(4) Park, K. H.; Fricker, A. Thermal inactivation and regeneration of technologically important enzymes. I. Peroxidase from horseradish and spinach. *Z. Ernahung-swiss.* **1977**, *16*, 81−91.

(5) Matsuda, H.; Hirayama, O. Enzymatic properties of a galactolipase from rice bran. *Agric. Biol. Chem*. **1979**, *43*, 697−703.

(6) Sahsah, Y.; Thi, A. T. P.; Roy-Macauley, H.; Arcy-Lameta, A.; Repellin, A.; Zuily-Fodil, Y. Purification and characterization of a soluble lipolytic acylhydrolase from Cow pea (*Vigna unguiculata L.*) leaves. *Biochim. Biophys. Acta* **1994**, *1215*, 66−73.

(7) Hirayama, O.; Oida, H. Changes of lipid and pigment compositions in spinach leaves during their storage. *J. Agric. Chem. Soc. Jpn.* **1967**, *43*, 423−428.

(8) Choe, E.; Park, K. H.; Kim, J. C.; Lee, S. H. Extraction and separation of lipids in freeze-dried spinach. Presented at the IFT Annual Meeting, 1997.

(9) Park, K. H.; Duden, R.; Fricker, A. Thermal inactivation and regeneration of technologically important enzymes. IV. Lipid-acyl-hydrolase in spinach. *Z. Ernahungswiss.* **1977**, *16*, 107−114.

(10) Heemskerk, J. W. M.; Bogemann, G.; Helsper, J. P. F. G.; Wintermans J. F. G. M. Synthesis of mono- and digalactosyldiacylglycerol in isolated spinach chloroplasts. *Plant Physiol.* **1988**, *86*, 971−977.

(11) Galliard, T. The enzymic deacylation of phospholipids and galactolipids in plants: purification and properties of a lipolytic acyl-hydrolase from potato tubers. *Biochem. J.* **1971**, *121*, 379−390.

(12) Pinsirodom, P.; Parkin, K. L. Selectivity of Celite-immobilized patatin (lipid acyl hydrolase) from potato tubers in esterification reactions as influenced by water activity and glycerol analogues as alcohol acceptors. *J. Agric. Food Chem*. **2000**, *48*, 155−160.

(13) Lee, F. A.; Mattick, L. R. Fatty acids of the lipids of vegetables. *J. Food Sci*. **1961**, *26*, 273−275.

(14) Pendlington, S. *1st Int. Congr. Food Sci. Technol.* **1962**, S 120 (Abstr.).

(15) Cotterell, R. C. Phospholipase A_2 from bee venom. *Methods Enzmol.* **1981**, *71*, 698−702.

(16) Kurioka, S. Synthesis of *p*-nitrophenylphosphorylcholine and hydrolysis with phospholipase C. *J. Biochem*. **1968**, *63*, 678−680.

(17) Abdelkarim, A.; Marcel, T.; Gardie, A. L.; Verger, R.; Noat, G. Phospholipase D from soybean suspension cultured cells. *Plant Cell Physiol*. **1995**, *36*, 989−996.

(18) Penheiter, A. R. Soybean root nodule acid phosphatase. *Plant Physiol*. **1997**, *114*, 597−604.

(19) Matsuda, H. Purification of a galactolipase from rice bran by affinity chromatography on palmitoylated gauze. *Agric. Biol. Chem*. **1979**, *43*, 463−469.

(20) Chang, B. S.; Park, K. H.; Lund, D. B. Thermal iactivation kinetics of horseradish peroxidase. *J. Food Sci*. **1988**, *53*, 920−923.

(21) Buttery, R. G. Importance of lipid derived volatiles to vegetable and fruit flavor. In *Flavor Chemistry of Lipid Foods*; Min, D. B., Smouse, T. H., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 156−165.

(22) Furuta, S.; Nishiba, Y.; Suda, I. A new method for judging deterioration of stored garden peas, an index of lipid peroxidation. *Biosci., Biotechnol., Biochem*. **1995**, *59*, 111−112.

(23) Oursel, A.; Escoffier, A.; Kader, J. C.; Dubacq, J. P.; Tremolieres, A. Last step in the cooperative pathway for galactolipid synthesis in spinach leaves: formation of monogalactosyldiacylglycerol with C18 polyunsaturated acyl groups at both carbon atoms of the glycerol. *FEBS Lett*. **1987**, *219*, 393−399.

(24) Clochard, A.; Guern, J. Destruction thermique De L'activite peroxidasique interpretation des curbes experiments. *Rev. Gen. Froid* **1973**, *8*, 860−870.

(25) Park, K. H.; Kim, Y. M.; Lee, C. W. Thermal inactivation kinetics of potato tuber lipoxygenase. *J. Agric. Food Chem*. **1988**, *36*, 1012−1015.

Received for review November 17, 2000. Revised manuscript received February 12, 2001. Accepted February 13, 2001. This study was supported by the Ministry of Agriculture and Forest, Korea, through a grant endowed to the Research Center for New Bio-Materials in Agriculture (RCNBMA) at Seoul National University and in part by the Brain Korea (BK) 21 project.

JF001379B