JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Purification and Characterization of Lipase in Buckwheat Seed

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To obtain basic information about enzymatic deterioration of buckwheat flour, triacylglycerol lipase (LIP; EC 3.1.1.3) was purified from buckwheat seed. The LIP consisted of two isozymes, LIP I and LIP II, and they were purified with purification folds of 60 and 143 with final specific activities of 0.108 and 0.727 μmol of fatty acid released per minute per milligram of protein at 30 °C using triolein as a substrate. Molecular weights were estimated to be 150 (LIP I) and 28.4 kDa (LIP I) by gel filtration and 171 (LIP I) and 26.5 kDa (LIP II) by SDS−PAGE. Optimal pHs of LIP activities were 3.0 (LIP I) and 6.0 (Lip II) using triolein as a substrate. Both LIP I and II reacted in the acidic pH range. Optimal temperatures were 30 (LIP I) and 40 °C (LIP II), and both LIP I and II were stable below 30 °C when *p*-nitrophenyl-laurate was used as a substrate. However, they were inactivated above 60 °C. On the other hand, when triolein was used as a substrate, optimal temperatures were 30 °C for both LIP I and II, and they retained 40% of their activity after a 4 h incubation of enzymes at 70 °C. LIP I and II had higher activity against triolein than monoolein or tri/monopalmitin. Most of the LIP activity was distributed in the embryo.

KEYWORDS: Buckwheat; lipase; purification; seed; fatty acid; lipid; organ distribution

INTRODUCTION

LIP catalyzes the first step of lipid catabolism. LIP is widely distributed in the plant kingdom and has been studied in many plant species (*1*, *2*). LIP have been characterized mainly in oilseed such as castor bean (*Ricinus communis*) (*3*), rape (*Brassica napus*) (*4*), and annual herb (*Vernonia galamensis*) (*5*). In these studies physiological roles of LIP were proposed for each plant species.

On the other hand, LIP is also an important concern in the food industry because lipid hydrolysis can cause deterioration of food quality (*6*). Buckwheat (*Fagopyrum esculentum* Moench) receives public attention as a healthy food. Buckwheat flour deteriorates more easily than wheat flour (*7*, *8*), and enzymatic activities are supposed to play an important role in the process of deterioration (*9*, *10*). Ohinata (1997) proposed that an accumulation of free fatty acids in buckwheat flour during storage is mainly caused by LIP (*10*). An increase in free fatty acids indicates a deterioration of the quality of buckwheat flour and will result in lipid peroxidation and deterioration of the flavor. Therefore, characterization of LIP in buckwheat flour is important to obtain basic information for quality control of buckwheat flour. However, the characteristics of LIP, such as isozyme composition, substrate specificity, thermal stability, and organ distribution, have not yet been clarified. In this study we purified and characterized LIP in buckwheat flour.

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MATERIALS AND METHODS

Plant Materials. Common buckwheat (*Fagopyrum esculentum* Moench var. Kitawasesoba) was grown at the experimental field of the National Agricultural Research Center for the Hokkaido region in Memuro, Hokkaido, Japan (longitude, 143°03′E; latitude, 42°53′N). Buckwheat seeds were sown June 6, 2002 and harvested in harvesting time (generally late August). Harvested seeds were dried at 40 °C for about 1 week and then threshed and stored at 4 °C until used for experiments.

Assay for in Vitro LIP Activity. LIP activity was determined using two procedures. First, the activity was determined spectrophotometrically using different *p*-nitrophenyl esters, *p*-nitrophenyl acetate (*p*NPC2), butyrate (*p*NPC4), caproate (*p*NPC6), and laurate (*p*NPC12) by the method of Winkler and Stuckman (1979, *11*) after minor modification. The assay mixture contained 0.5 mM of each *p*-nitrophenol ester in a 200 mM acetate−NaOH buffer (pH 6.0) containing 0.3% Triton X-100 and 4% (v/v) acetone in a total volume of 0.3 mL. Assay was initiated by addition of the enzyme. Activities were determined at 22 °C by measuring the initial rate of increase in absorbance at 400 nm.

Second, the activity was determined by measuring the concentration of fatty acid hydrolyzed from tri/monoolein or tri/monopalmitin. The assay mixture was a 200 mM buffer (citrate−NaOH pH 4.0 for LIP I and acetate−NaOH pH 5.0 for LIP II) containing 0.3% Triton X-100, 4% (v/v) acetone, and 100 mM substrate (triolein, monoolein, tripalmitin, or monopalmitin dissolved in hexane) in 5% (w/v) arabic gum in a total volume of 0.3 mL. Assay was initiated by addition of the enzyme. The reaction was performed using an air incubator with shaking (30 °C, 300 rpm) for 4 h. After incubation, 1 mL of ethanol was added to samples to extract fatty acid. (The extraction efficiency of fatty acid was above 95% in this procedure.) The fatty acid was analyzed directly by HPLC using an ODS column (Capcell PAK C18 UG120 S5,

10.1021/jf049271+ CCC: $27.50
Published on Web 11/02/2004

4.6 mmφ × 250 mm; Shiseido Japan) with isocratic conditions at 40 °C at a flow rate of 1.5 mL/min using acetonitrile/0.1% phosphate (8:2) and detected by UV at a wavelength of 210 nm (experimental protocol can be obtained on the Shiseido Corp. homepage (http://www.shiseido.co.jp/hplc-bin/e/index.asp), in which you can become a member of the Shiseido HPLC Data Service). The free-fatty acid concentration was determined by a standard curve derived from standard free-fatty acid (oleic acid or palmitic acid). One unit of the LIP activity was defined as the amount of enzyme activity that released 1 μmol of fatty acid released per minute. Because tri/monoolein or tri/monopalmitin contains a small amount of free fatty acids, we extracted them from tri/monoolein or tri/ monopalmitin with ethanol 3 times prior to preparing a substrate.

Optimal pH, Optimal Temperature, and Thermal Stability of Enzyme Activity. The optimal pHs of enzyme activity was determined at 22 °C at pHs ranging from 2.0 (200 mM glycine−HCl buffer), 3.0−4.0 (200 mM citrate−NaOH buffer), 5.0−6.0 (200 mM acetate−NaOH buffer), 7.0 (200 mM phosphate−NaOH buffer), 8.0 (200 mM tris−HCl buffer), 9.0−10.0 (200 mM borate−NaOH buffer) using *p*NPC12 or triolein as a substrate.

The optimal temperature for LIP activity was determined by measuring it at 10−70 °C using *p*NPC12 or triolein as a substrate. Reaction mixtures were preincubated at each temperature for 15 min prior to measurement of the LIP activity. The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity.

To determine thermal stability, enzyme solutions were incubated at 10−70 °C for 4 h prior to assay, and then LIP activity was assayed at 22 °C using *p*NPC12 or triolein as a substrate. The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity.

Purification of LIP. Two hundred grams of buckwheat seeds were ground to a powder using a mortar. The powder was homogenized for 2 h with 2000 mL of buffer, which contained 50 mM acetate−NaOH buffer (pH 5.0), 1 mM EDTA, and 1 mM fresh PMSF. A crude enzyme solution was obtained by centrifugation and then precipitated with 0−80% saturation of solid $(NH_4)_2SO_4$. To increase the purification fold, the solution was divided into eight portions before all the steps below were carried out. The precipitate was dissolved in buffer A, which contained 50 mM acetate−NaOH buffer (pH 5.0) and 1 mM EDTA, and dialyzed overnight against buffer A. The dialyzed enzyme solution was centrifuged and applied to a DEAE-Sepharose column (2.4 × 11.5 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. The LIP was eluted using a linear 600 mL gradient of 50−400 mM NaCl in buffer A. Active fractions were collected and loaded onto a Sephacryl S-200 column (2.4 × 66 cm, Amersham Pharmacia Biotech) equilibrated with buffer A. Active fractions were collected and stored at −30 °C until they were used. In every purification step the LIP activity was measured using *p*NPC12 as a substrate. All the above steps were carried out at 4 °C.

Organ Distribution of LIP. The embryo, testa, and endosperm of 20 common buckwheat seeds were separated carefully using tweezers after removal of the panicles. Each separated organ was homogenized with buffer, which contained 50 mM acetate−NaOH buffer (pH 5.0), 1 mM EDTA, and 1 mM fresh PMSF for 20 min. A crude enzyme solution was obtained by centrifugation, and then LIP activity was measured using *p*NPC12 as a substrate.

Protein Determination, Electrophoresis, Gel Staining, and Determination of Purification Rate. Total soluble protein concentrations were measured by the method of Bradford (*12*) using bovine serum albumin as a standard. The SDS−PAGE was carried out using 10% polyacrylamide gels (*13*). Then gel was stained using Silver Stain Kit Wako (Wako Pure Chemical Industries, Ltd.). The Purification rate was derived by calculation (LIP bands intensity/total bands intensity × 100). Band intensity was quantitated using a densitometer following gel staining.

RESULTS AND DISCUSSION

Purification and Molecular Weight of LIP. We purified the LIP from buckwheat seed (**Figure 1**; **Table 1**). The LIP consisted of two isozymes, LIP I and LIP II, and they were separated by ion-exchange chromatography. The molecular weights of LIP I and II were 150 and 28.4 KDa by SDS−PAGE and 171 and 26.5 KDa by gel filtration, respectively (**Table 2**). From these values it appears that LIP I and II are monomers. The molecular weights of LIP II are close to the rice (*Oryza Sativa*) lipase II (32KDa) (*14*) and annual herb lipase (30KDa) (*5*). Purification rates were 72% (LIP I) and 95% (LIP II) indicating near homogeneity (**Figure 1**). In some cases LIP actively aggregated or became insoluble (*10*−*12*). However, in this experiment aggregation was not observed. In addition, extraction efficiency was not increased using Tween-20 and Triton X-100. Through the purification steps no other obvious LIP activities were detected except LIP I and II. From these results we assume that LIP I and II are the major LIPs in buckwheat seed. The final specific activities of LIP I and II were 0.360 and 0.858 μmol of *p*-nitrophenol released per minute per milligram of protein at 22 °C when *p*NPC12 was used as a substrate (**Table 1**). These values were very low compared with *Candida rugosa* Lip2 and LIP 3 (179 and 298 μmol of *p*-nitrophenol released per minute per milligram of protein) (*15*). When triolein was used as a substrate, final specific activities were 0.108 and 0.727 U per milligram of protein at 30 °C (**Table 1**). Generally, lipase activities in plant seeds are usually below 0.1 U per milligram of protein (*16*). The specific activity of LIP II is higher than them, whereas it is much lower than other

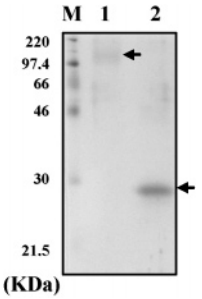

Figure 1. SDS−PAGE of the purified LIP isozymes. Purified LIP isozymes and molecular weight marker were subjected to 10% SDS−PAGE (lane M, molecular weight marker; lane 1, purified Lip I; lane 2, purified Lip II, then stained using "Silver Stain Kit Wako" (Wako Pure Chemical Industries, Ltd.)).

Table 1. Purification of the LIP I and LIP II from Buckwheat Seeds

	purification step	total protain, mg	specific activity,[a] μmol/min/mg protein	yield, %	fold
	crude extract	29200.00	0.006	100.0	1.0
	concentration	13600.00	0.006	48.2	1.0
LIP I	DEAE-Sepharose	23.50	0.156	2.1	26.1
	Sephacryl S-200	8.16	0.360 (0.108)[b]	1.7	60.2
LIP II	DEAE-Sepharose	47.20	0.267	7.2	44.6
	Sephacryl S-200	4.72	0.858 (0.727)[b]	2.3	143.0

[a] Enayme activities were determined using *p*NPC12 as a substrate. [b] Triolein was used as a substrate.

Table 2. Molecular Weight of LIP I and LIP II[a]

	SDS-PAGE	gel filtration
LIP I	150000 ± 9600	171000 ± 7900
LIP II	28400 ± 360	26500 ± 1000

[a] Data are means ± SD of three measurement by SDS−PAGE and five measurements by gel filtration.

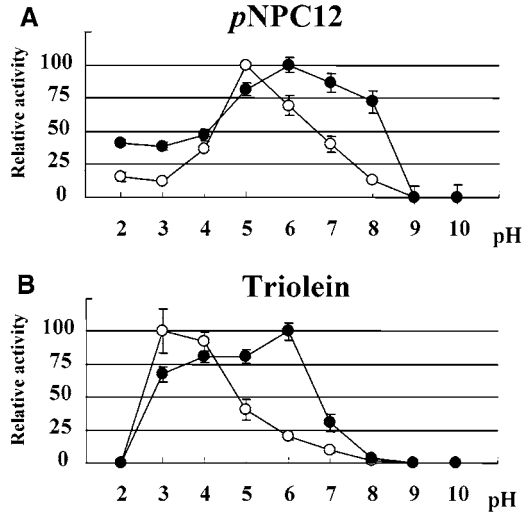

Figure 2. Optimal pH of LIP. The optimal pHs of Lip I (○) and II (●) were measured using *p*NPC12 (**A**) or triolein (**B**) as a substrate. LIP activity was measured at different pHs at 22 (*p*NPC12) or 30 °C (triolein). Activities are expressed as relative specific activities. Data are the means of three independent experiments. Bars indicate SD.

lipases such as that from *Euphorbia characias* latex (1500 U per mg protein) (*16*) or yeast lipase (*Candida rugosa)* (*15*).

Optimal pH of LIP. The optimal pH was determined using *p*NPC12 and triolein as substrates (**Figure 2**). When *p*NPC12 was used as a substrate (**Figure 2A**), the optimal pH was 5.0 (LIP I) and 6.0 (LIP II). Both LIP I and II reacted between pH 2.0 and 8.0 and did not react above pH 9.0. On the other hand, when triolein was used as a substrate (**Figure 2B**) the optimal pHs were 3.0 (LIP I) and 6.0 (LIP II). Both LIP I and II reacted between pH 3.0 and 7.0 and did not react below pH 2.0 and above pH 8.0. LIP I reacted in a narrower pH range than LIP II did. Both LIP I and II had high activity in the acid pH range. The pH of buckwheat flour is generally around pH 6.8. Therefore, the pH of buckwheat flour is suitable for reaction with LIP. Further, 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 (**Figure 2B**). Optimal pHs of LIP I and LIP II are different from rape lipase (*2*), mustard lipase (*Sinapis alba*) (*2*), lipase from cotyledons of lupine (*Lupinus albus*) (*2*), and rice lipase I and II (*14*), in which optimal pHs were between 8 and 9. On the other hand, optimal pHs of LIP I and II are very similar to castor bean acid lipase (*3*) in which the optimal pH was 4.3.

Optimal Temperature and Thermal Stability of LIP. The optimal temperature and thermal stability were investigated (**Figure 3**).

When *p*NPC12 was used as a substrate, patterns of LIP I and II activity about optimal temperature (**Figure 3A**) and thermal stability (**Figure 3C**) were roughly the same. The optimum temperatures were 30 (LIP I) and 40 °C (LIP II), and neither LIP I nor II reacted above 70 °C (**Figure 3A**). Both LIP I and II were stable below 30 °C and completely inactivated above 60 °C (**Figure 3C**).

When triolein was used as a substrate, patterns of LIP I and II activities about optimal temperature were different; LIP I had an activity in a wider range than LIP II (**Figure 3B**). The optimum temperature was 30 °C for both LIP I and II, and they had activity even at 70 °C (**Figure 3B**). On the other hand, thermal stability was roughly the same (**Figure 3D**). Both LIP I and II were stable below 30 °C and retained activity at 70 °C (**Figure 3D**). Both LIP I and II had about 50% of the activity at 10 °C as at 30 °C (**Figure 3B**). Therefore, LIP would react during storage even if stored at 10 °C.

Substrate Specificity of LIP. To characterize LIP I and II the substrate specificity of LIP I and II was investigated using different *p*-nitrophenyl esters and tri/monoolein or tri/ monopalmitin as a substrate (**Figure 4A**,**B**). When *p*-nitrophenyl esters

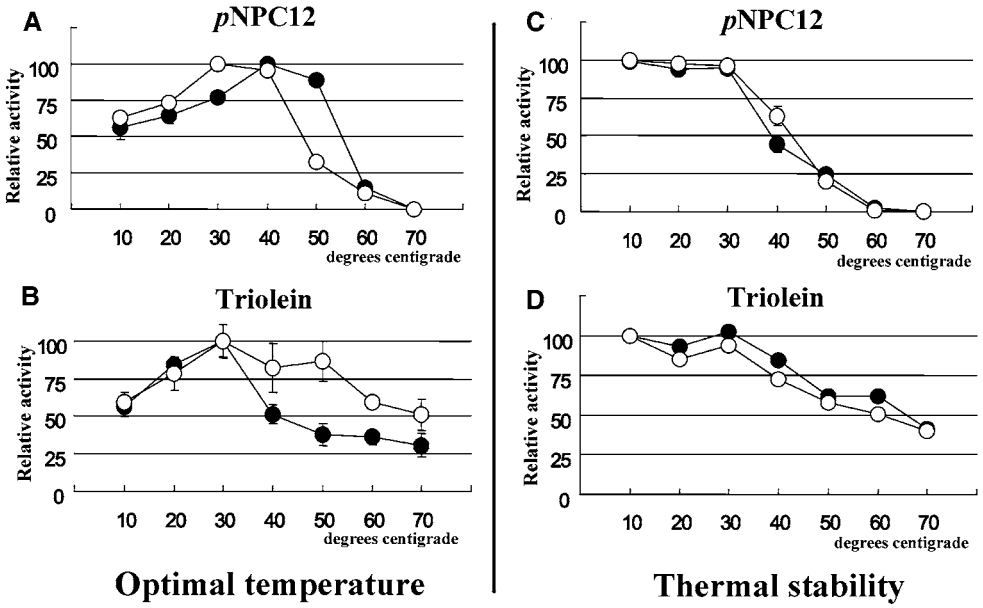

Figure 3. Optimal temperature and thermal stability of LIP. Optimal temperature of Lip I (○) and II (●) were measured using *p*NPC12 (**A**) or triolein (**B**) as a substrate. Lipase activities were measured at different temperature. The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity. Data are the means of three independent experiments. Bars indicate S.D. Thermal stabilities of Lip I (○) and II (●) were measured using *p*NPC12 (**C**) or triolein (**D**) as a substrate. Enzyme solutions were incubated at 10–70 °C for 4 h prior to assay, and then LIP activity was assayed at 22 (*p*NPC12) or 30 °C (triolein). The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity. Data are the means of three independent experiments. Activities are expressed as relative specific activities. Data are the means of three independent experiments. Bars indicate SD.

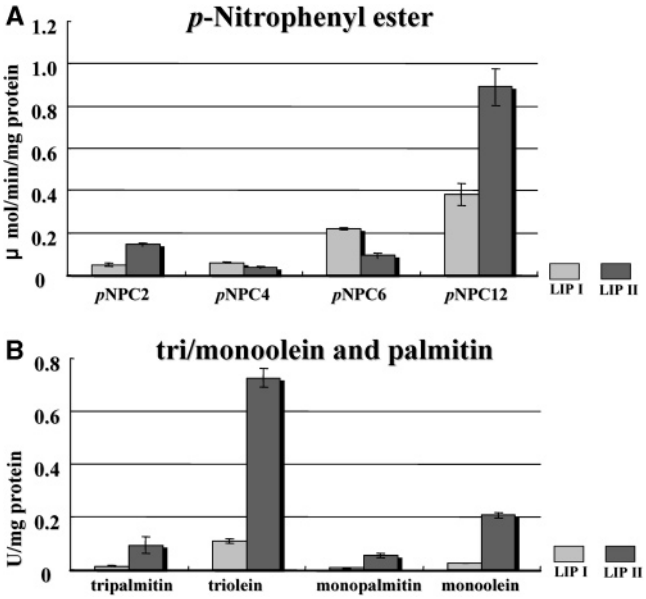

Figure 4. Substrate specificity of LIP. (**A**) LIP activity was measured using a different *p*-nitrophenyl ester as a substrate at 22. The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity. Data are the means of three independent experiments. Bars indicate S.D. (**B**) LIP activity was measured using tri/monoolein or tri/monopalmitin as a substrate. One unit of the LIP activity was defined as the amount of enzyme activity that released 1 μmol of fatty acid released per minute. The composition of the reaction mixture was the same as that described in Assay for in Vitro LIP Activity. Data are the means of three independent experiments. Bars indicate SD.

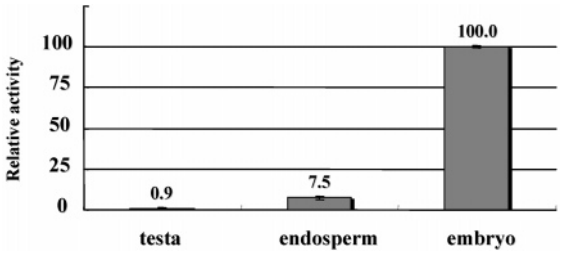

Figure 5. Organ distribution of LIP activity in buckwheat seed. The LIP activity of the embryo, testa, or endosperm of 20 buckwheat seeds was measured. LIP activity was measured using *p*NPC12 as a substrate at 22 °C. Activities are expressed as relative activities on organ base. Data are the means of three independent experiments. Bar indicates SD.

were used as a substrate, the specific activity for each ester differed between LIP I and II (**Figure 4A**). LIP I activity tended to be stronger as the chain length became longer, and LIP II activity followed a similar pattern. This result is similar to *Candida rugosa* Lip2 and Lip3 (*15*). LIP II activity for *p*NPC12 was much higher than for others. In buckwheat seed most LIP activity was distributed in the embryo (**Figure 5**). This finding is consistent with the organ distribution of lipoxygenase protein (and peroxidase activity (our unpublished results)), which should affect fatty acid metabolism. In addition, Dorrell (1970) reported that about 60−70% of the oil was also distributed in the embryo. The fatty acid compositions of the lipid of palmitic acid, oleic acid, and linoleic acid were roughly 17%, 36%, and 33%, respectively. These results indicate that these fatty acid species can be produced by LIP activity in buckwheat flour during storage or in germinating seed. From these findings we investigated the substrate specificity of LIP I and II using tri/monoolein or tri/monopalmitin as a substrate (**Figure 4B**). Monoolein and monopalmitin may be poor substrates for lipase. However, investigations of substrate specificity of them are important to study roles of lipase activity concerning quality deterioration of buckwheat flour, because in the process of hydrolyzing triolein or tripalmitin by lipase activity, monoolein or monopalmitin should be generated. Substrate specificities of LIP I and II took roughly the same pattern against tri/ monoolein or tri/monopalmitin; activities were higher in the order triolein > monoolein > tripalmitin > monopalmitin. Both LIP I and II had higher activity against triolein than against monoolein and was higher against tripalmitin than against monopalmitin. From this result LIP activity should be higher against triacyl glycerol than monoacyl glycerol. LIP II had about two times the specific activity of LIP I in all substrates tested. On the basis of these results LIP I and II had different substrate specificities.

Buckwheat flour tends to deteriorate easily, and LIP activity is supposed to play an important role in the deterioration. To inactivate LIP activity in buckwheat flour, heat treatment would be effective. However, heat treatment is costly and would result in deterioration of flavor and color. Therefore, breeding of a buckwheat cultivar with low-LIP activity is desirable. Further, to develop such a cultivar, it is important to clarify which isozyme is more important for the quality of buckwheat flour.

ACKNOWLEDGMENT

We thank T. Saruwatari and M. Oizumi for their technical assistance.

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Received for review May 7, 2004. Revised manuscript received September 7, 2004. Accepted September 15, 2004.

JF049271+