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REVIEW

Purification, Distribution, and Characterization Activity of Lipase from Oat Seeds (*Avena sativa* L.)

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Abstract Plant lipases have been chiefly studied as an esterase for hydrolyzation of triacylglycerol (a true lipase), which supplies energy for seed germination. Lipases are widely distributed in plants, animals, insects, and microorganisms. However, recent studies suggest that plant lipases have physiological functions other than triacylglycerol hydrolysis. In the present study, a plant lipase that has enzyme properties distinct from those of a true lipase was purified and characterized from oat seedlings. The lipase was purified 189-fold to a 0.53% purification ratio with high specific activity (34.656 U/mg). Analysis of the protein by Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a homogenous purified lipase. The lipase had higher enzyme specificity to monoacylglyceride and short chain fatty acids. Synthesis of the lipase was active at an early stage of germination for 6 days and decreased thereafter. Most of the lipase was found in the upper part of the oat seedling excluding the root. Within the young leaves, the lipase is located only in vessels and sieve tubes. However, infection of a pathogen, *Pseudomonas syrinae* pv. *oryzae,* elevated the lipase synthesis. In addition, the lipase had an ability to hydrolyze *E.coli* lipopolysaccharide. These results suggested that oat lipase may play a physiological role in defense against pathogens.

Keywords characterization · distribution · infection · lipase · pathogen · purification · oat

Introduction

Lipases (EC 3.1.1.3) are ubiquitous complicated enzymes with unrivalled potential in the emerging areas of biotechnology, food technology, and biomedical sciences (Reis et al., 2009). They are primarily responsible for the hydrolysis of acyl glycerides during lipid processing and principally catalyze the hydrolysis of ester bonds in mono-, di- and triacylglycerols (TAG) (Patel et al., 1996; Hasan et al., 2009; Vijayakumar and Gowda, 2013). Lipases from microorganisms thus far have been the most studied lipases; however, other sources of lipases include plants and animals (Hasan et al., 2006). In the case of plants, lipases are mostly present in oilseeds and laticifers (Rivera et al., 2012). Plant lipases have interesting biochemical properties such as pH and temperatures (Bhardwaj et al., 2001). The isolation and purification of plant lipases are carried out with relatively simple techniques (Fuchs et al., 1996; Klose and Arendt, 2012; Rivera et al., 2012). Plant lipases have many potential applications in a laboratory scale. As an example, plant lipases have proven to be highly specific for fatty acids present within the plant; this feature can be exploited in biotechnological applications (Hasan et al., 2006; de Sousa et al., 2010). Plant lipases have two major functions: providing energy by hydrolyzing the oils stored in the seeds and protection (e.g., as antipathogenic agents), as in the case of laticifer lipases and some lipases from *Arabidopsis* (Kwon et al., 2009; Lee et al., 2009). Plants have both passive and active defenses against pathogen attack (Koeck et al., 2011). Pathogen ingress to host tissue is the first critical step in infection, as nutrients on the leaf surfaces are believed to be very limited. In the case where pathogens breach passive defenses, plants rely on active immunity to restrict pathogen proliferation (Gimenez-Ibanez and Rathjen, 2010). Defense mechanisms have been reported in plants where lipases and other enzymes directly attack microorganisms that enter the area of the pistil and stamen through direct contact (Konno, 2011). Previous studies suggested that *Arabidopsis* AtGLIP1 directly attacked necrotrophic *fungus Alternaria sp. brassicola* to reduce the strength of the cell membrane and cell wall as a direct anti-microbial action (Oh et al., 2005). However, the function of lipases in defense mechanisms is not yet fully understood.

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In the present study, we have separated lipase from oats and demonstrate the characteristics of the lipase at early germination stage, and distribution of lipase in tissue was studied to infer the function of the plant lipase. Furthermore, the substrate specificity of the lipase and its involvement in infection with pathogens were examined in order to understand the functions of lipase associated with pathogen resistibility.

Materials and Methods

Materials. Oats (*Avena sativa L.*) were purchased from Maine Potato Growers Inc. (USA). Pheny-Sepharose CL-4B and carboxymethyl (CM)-Cellulose were purchased from Sigma (USA). Ammonium sulfate was purchased from United States Biochemical (USA). All reagents and chemicals were of analytical grade.

Cultivation of oat seeds. Oat seeds were immersed for 2 h under darkness at 28°C and were evenly scattered and incubated on an aluminum plate (40×55 cm) with damp vermiculites. Vermiculites were covered over seeds in such a way that seeds could hardly be observed. Water was sprinkled once a day at 27 to 28°C under darkness to maintain humidity. The seeds were then incubated for 4 to 5 days. When the seeds germinated and grew to about 5 cm, the upper 3 cm of the seedling was harvested and used as a sample. All oat sowing and harvesting processes were carried out under a green safety light.

Purification of a lipase from oat seedlings. Purification of a lipase from oat seedlings followed the procedure described by Park et al. (2012). Briefly, the seedlings were extracted with an extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% glycerol, 14 mM β-mercaptoethanol, 4 mM phenylmethylsulfonyl fluoride, 0.02% sodium azide, and 0.5 $\mu g/mL^{-1}$ leupeptin), and treated with 0.1% polyethyleneimine followed by precipitation with 50% ammonium sulfate. The precipitant was dissolved in buffer A (50 mM Tris, pH 7.5, 5 mM EDTA, 14 mM β-mercaptoethanol and 2 mM PMSF), desalted by a Sephadex G-25 column, and passed through a phenyl-Sepharose CL-4B column and then through CM-cellulose column. The protein bands of each elute were identified on a 10% SDS gel stained with silver nitrate, and used for the enzyme activity assay.

Spectrophotometric assay with *p*-nitrophenyl palmitate (*p*-NPP). Lipase activity was measured with a spectrophotometric assay at 25°C with *p*-nitrophenyl palmitate (*p*-NPP). To prepare the reaction mixture, the substrate stock solution (10 mL isopropanol containing 30 mg *p*-NPP) and buffered solution (0.2 M sodium phosphate buffer, pH 7.0, 207 ml Na-DOC, and 100 mg gum arabic) were mixed to 1:9 ratio. The reaction mixture was incubated for 10 min at 35°C in a water bath. Before the measurements, the reaction was stopped by the addition of 2 M $NaCO_3$. To evaluate the activity, the increase of absorption at 410 nm was followed with a Pye Unicam SP8-100 spectrophotometer for 10 min.

Electrophoresis and silver staining. The electrophoresis was transformed using the method of *Laemmli*. The SDS-sample buffer [0.125 M Tris-HCl (pH 6.8), 5% (w/v) SDS, 30% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, and 0.002% (w/v) bromphenol blue] was mixed with sample protein and then boiled for 3 min. The electrophoresis was carried out for 2 h, and the protein separation was confirmed by staining for 40 minutes using a staining solution [0.1% (w/v) Coomassie brilliant blue R-250 solution : methanol : accetic acid=5:5:2, v/v/v] followed by discoloring using a decolorization solution [30% (v/v) methanol, 10% (v/v) acetic acid, 1% (v/v) glycerol]. For confirmation of a small amount of protein fractions, a silver ($AgNO_3$) staining method was conducted. After the sample was treated by SDS gel electrophoresis, the gel was mixed with a fixing solution (methanol : acetic acid : dH_2O=40:10:50, v/v/v) and stirred for 30 min to immobilize the protein fractions. The protein fractions were then washed for 15 min twice a day using a cleaning solution (methanol : acetic acid : dH_2O=10:5:85, v/v/v). After washing, the gel was incubated in a solution containing 3.4 mM potassium dichromate and 3.2 mM nitric acid for 15 min. The gel was washed twice with distilled water, treated with 12 mM silver nitrate solution for 15 min, and then washed twice with distilled water. The protein fractions were developed in a solution of 0.28 M sodium carbonate containing 0.02% (v/v) formaldehyde. They were then transferred to a solution of 3% (v/v) acetic acid, and were finally fixed.

Western blotting. After electrophoresis, the gel was soaked in a transfer buffer of 124 mM Tris-Cl, 192 mM glycine, and 20% methanol. The gel was then placed in 100% methanol for 15 seconds; moved to sterile water for 2 minutes; and finally placed in a transfer buffer for five minutes for equilibration. A transfer membrane (Immobilon Millipore pore size, 0.45 μg; filter type: PVDF) soaked in the transfer buffer was overlaid on top of this gel. After transfer, the membrane was washed in a washing buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20). In order to prevent nonspecific proteins from binding to the membrane, the membrane was put into a shaker for 2 hours for the blocking process using a blocking buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, and 2.5% skim milk). Primary antibody was diluted with an incubation buffer (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, and 1.0% skim milk) to a 1/500 ratio. It was then stirred for 1.5 h, and subsequently washed with a washing buffer. The secondary antibody was next diluted with the incubation buffer to a 1/2000 ratio, stirred for 1.5 h, and then washed with the washing buffer. Finally, the membrane was soaked in the detection solution. After the membrane completely dried out, it was exposed to an X-ray film for 15 seconds and then developed.

Immunohistochemistry. After being placed in 60°C dry oven four times for 5 min each time in a solution of xylene, the tissue was treated two times for 5 min each in a solution of 100% ethanol, two times in a solution of 95% ethanol, and two times in a solution of 70% ethanol, and then left standing for 10 minutes under running water. Using sterile water, the tissue washed twice

and treated with TBST. It was subsequently left for 20 min at room temperature in a methanol solution containing 0.6% H_2O_2. The sample was washed and subjected to blocking for 40 minutes using a TBS buffer containing 1% BSA to treat the primary antibody followed by washing with TBS to incubate the secondary antibody for 50 minutes. Subsequently, the tissue was washed again with TBS, the sample tissue was then incubated for 30 minutes at room temperature, and then washed with TBS. The process of DAB discoloration was observed for 5 minutes, in sterile water, followed by treatment of TBS, haematoxlin, sterile water, ethanol, and xylene.

Lipase activity measurement by gas chromatography. Lipase activity was assayed *in vitro* with gas chromatography. The assay mixture containing 10 mM monopalmitate in acetonitrile, 40 μL ethanol, lipase 10 μL, and 50 mM Tris-HCl 940 μL was incubated in a water bath at 50°C for 1 h with stirring. The reaction mixtures were extracted with 5 mL of chloroform and dried in a SpeedVac centrifugal concentrator. The residue was suspended in hexane (500 mL) and analyzed for free fatty acids using gas chromatograph (HP, 6890 GC FID system).

Results

Isolation of lipase from oat seedling. Oats have stronger resistance to microorganisms as compared to other crops. Oats, widely recognized as a healthy food, contain a lipase that is more active than those of other cereals (Cai et al., 2012). Oat seedling was extracted, and 40% ammonium sulfate was precipitated, as described in Materials and Methods. The lipase was partially purified by phenyl-Sepharose column chromatography after being dissolved in 450 mL of Tris buffer solution. Lipase activity was identified by 85 fractions in total protein extracts (Fig. 1A). The sample was electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. Results of protein isolation are shown in Fig. 1B. The fraction that shows lipase activity was determined through phenyl-Sepharose column chromatography followed by CM-cellulose chromatography. The protein was then eluted with the NaCl density gradient elution method (Fig. 1C). Protein containing lipase activity was identified from 83 to 107 fractions, and the isolated protein was confirmed using SDS-PAGE (Fig. 1D). These results suggest that lipase is a

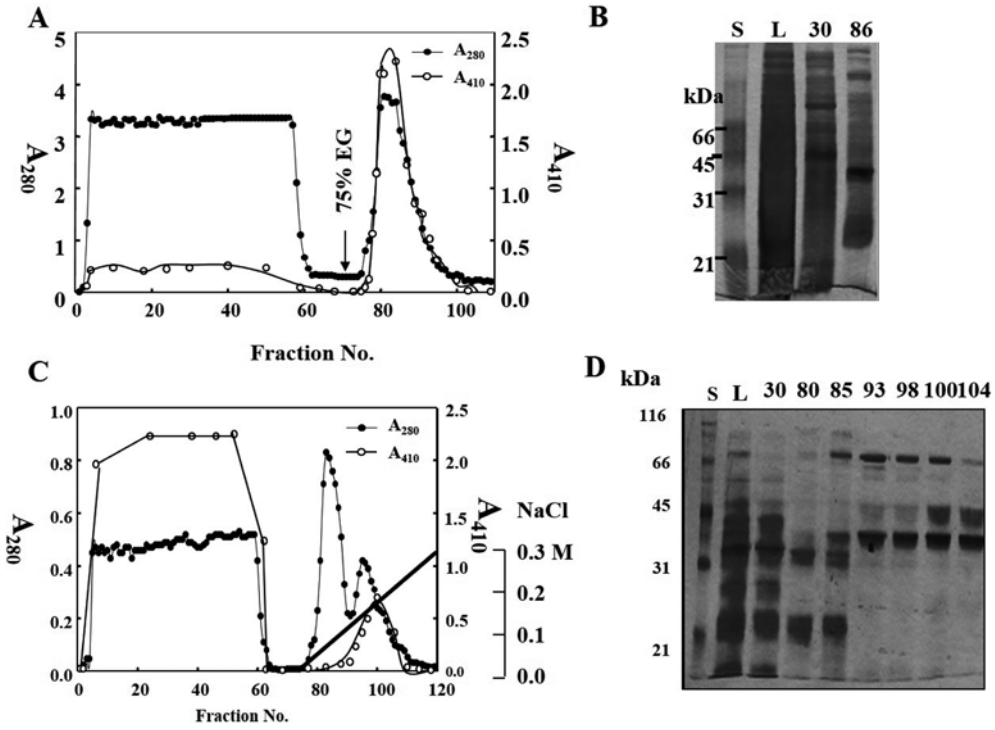

Fig. 1 Isolation of lipase from oat seedling. (A) Phenyl-Sepharose chromatography of total protein extract from oat seedlings. Oat extracts were precipitated by PEI and ammonium sulfate, dissolved in 20 mM Tris buffer, and applied on the phenyl Sepharose CL-4B column (3.8× 5.5 cm). The bound proteins were eluted with 75% ethylene glycol in 20 mM Tris buffer at a flow rate of 80 ml per hour. Fractions of 10 ml eluant were assayed for protein (absorbance at 280 nm) and for lipase activity (absorbance of p-NPP at 410 nm). (B) SDS-PAGE of phenyl-Sepharose column fractions. The sample was electrophoresed on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. S: Size marker; L: Loading; 30 and 86: Fraction No. (C) CM-Cellulose chromatography of the lipase solution from phenyl-Sepharose column. Lipase-active fraction was applied on a CM-Cellulose column (2.2×6.0 cm). After washing with 20 mM Tris-HCl (pH 7.5) buffer, the bound proteins were eluted with a linear gradient of 20 mM Tris-HCl (pH 7.5) and 0.3 M NaCl in 20 mM Tris-HCl (pH 7.5) at a flow rate of 20 mL per hour. The eluates were collected by 3 mL per fraction. (D) SDS-PAGE of CM-Cellulose column fractions. The eluants from the CM-Cellulose column were electrophoresed on 10% SDS-polyacrylamide gel and stained with silver nitrate. S; size marker, L; Loading, No. 30-104; fraction No.

Table 1 Purification of lipase from oat seedlings

	Protein (mg/mL)	Total activity Unit*	Specific activity Unit*	Purification Fold	Yield (%)
Crude extract	3,577.69	3,662	0.183	1.00	100.00
PEI Precipitation	2,988.34	4,442	0.219	1.20	83.52
50% Ammonium sulfate Precipitation	1,817.23	7,187	0.360	1.97	50.79
Phenyl-Sepharose Chromatography	176.27	204	3.716	20.30	4.93
CM-Cellulose Chromatography	18.90	5.5	34.656	189.30	0.53

Lipase activity was measured by the absorbance of *p*-nitrophenol produced from *p*-NPP by the lipase at 410 nm.
*One unit: one μm palmitate produced from *p*-NPP for 1 min.
*Specificity: unit/mg protein

Table 2 Substrate specificity of the purified oat lipase

Substrate	Specific activity (U/mg)	Relative activity (%)
The number of chain		
Monopalmitate	598	100
Dipalmitate	86	14
Tripalmitate	11	2
The length of chain		
Monobutylate (C4)	755	100
Monolauric acid (C12)	656	87
Monopalmitate (C16)	554	73

Purified lipase hydrolyzes glycerol mono-, di- and tri-palmitates and monoacylglycerol with different chain lengths. Activity was assayed by GC. Relative activity was expressed as the percentage of the activity towards monopalmitate and monobutylate.

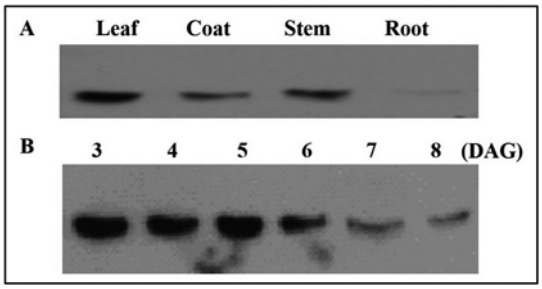

Fig. 2 Western blots of the lipase from different oat tissues and 6 days after germination (DAG). After manufacturing the antibody of the lipase, electrophoresis and Western blot were performed. (A) Expression of lipase from the oat seedlings in leaves, stems, coat, and roots. Tissues were taken from 4 day-old seedlings. (B) Expression of lipase depending on time (day) in seedlings.

40 kDa protein. Purification efficiencies from crude extracts to isolation are summarized in Table 1; 5.51 mg of lipase was isolated from 3 kg of oat seedlings. After purification, the lipase was purified 189-fold to a 0.53% purification ratio with high specific activity (34.656 U/mg). The lipase (carboxylesterase) was purified to a homogeneity on SDS-gel and partially sequenced for its amino acid sequences. The lipase was a 40 kDa monomeric protein composed of 358 amino acids belonging to the GDSL family with SAG motif.

Lipase activity and substrate specificity. We investigated the substrate specificity of isolated and purified lipase from oat seedlings using various lipid substrates. The isolated lipase showed specific activity in monopalmitate, whereas lipase activity was weak in dipalmitate and tripalmitate (Table 2). The lipase activity of the short chain carboxylic acid (butylate) was higher than that of the long chain carboxylic acid (palmitate). In addition, the 1- and 3- chains were separated well, but the 2- chain was not. Consequently, isolated lipase showed higher specific activity in short chain fatty acids than in long chain fatty acids. This suggests that the activity of carboxylesterase was higher than that of TAG hydrolase and lipase from oat seedlings, which plays roles in transesterification and esterification. Therefore, isolated lipase may be classified as an esterase or acyltransferase in lipase type II rather than as a TAG lipase.

Western blots of the lipase from different oat tissues. After extraction of lipase from the leaves, coat, stem, and roots, tissue-specific expression of the lipase were investigated. Lipase from the oat seedlings was found in leaves, stems, and coat, but not in the roots (Fig. 2A). In order to investigate the lipase-specific expression during the growing periods, the upper 20 mm of the seedlings were cut each day. The lipase was extracted daily, and electrophoresis and Western blot were performed. The lipase was expressed actively at an early stage of germination for 6 days and rapidly disappeared thereafter (Fig. 2B).

Immunohistochemistry of the lipase within the oat stem tissue. In order to study the location of the lipase in organelles, immunohistochemistry was performed. Lipase was stained with DAB (yellow color) in vascular tissue containing vessels and sieve tubes. From the results, lipase was found to be distributed primarily in the vessels and sieve tubes, suggesting that lipase was transferred to the vessels or sieve tubes when stimulate externally (Fig. 3).

Western blot of the lipase from oat seedlings treated with *Psudomonas syringae* pv *oryzae*. Regarding the role of lipase in the plant, it was proposed that lipase is a producer of signal substances in resistance signaling pathways of pathogen penetration. In order to identify anti-pathogenic effects of the lipase, changes in lipase expression were examined by Western blotting. After oat seeds were grown in the dark for 4 days under same condition: group A (no treatment) and groups B and C (cut at the upper part

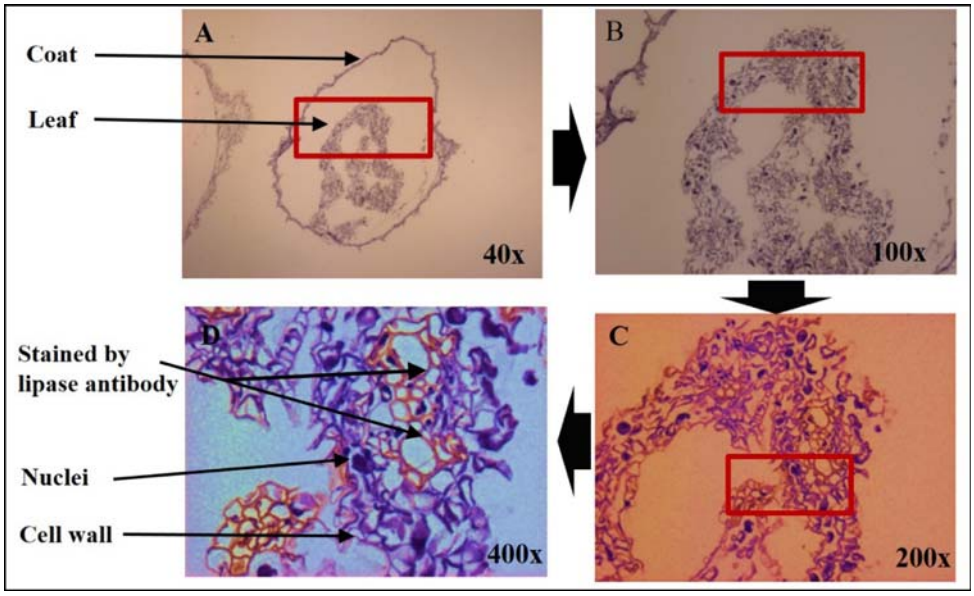

Fig. 3 Immunohistochemistry of the lipase within the oat stem tissue. Oat stem tissue was sectioned in the transverse direction. A, B, C, and D are 40×, 100×, 200×, and 400× magnification, respectively. Lipase was stained with DAB (yellow color) in vascular tissue containing vessels and sieve tubes.

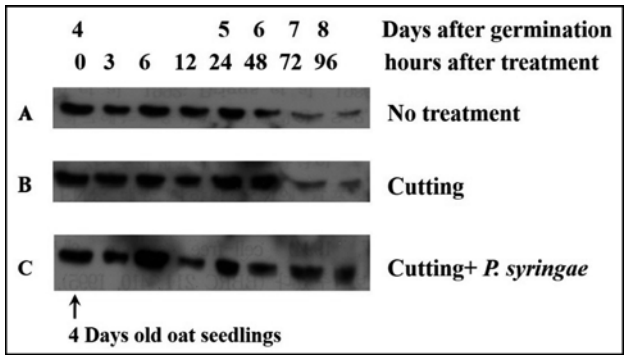

Fig. 4 Western blot of the lipase from oat seedlings treated with *Psudomonas syringae* pv *oryzae.* (A) 4-day-old seedling shoots, no treatment, (B) cut at the upper part of the seedling, and (C) the cut shoots were sprayed with *Psudomonas syringae* pv *oryzae*. After the treatments, the samples were incubated for indicated times.

(0.5 to 1 cm) of the seedling). Group C was sprayed with *Psudomonas syringae* pv *oryzae* at the upper part of the seedling. After growing in the dark for 4 days, the expression level of lipase was measured. Whereas the amount of lipase expression decreased in groups A and B after 48 h, in group C, which was treated with pathogens, the lipase was continuously expressed after 96 h (Fig. 4).

The hydrolysis of *E. coli* lipopolysaccharide using lipase. To find evidence that the lipase is associated with pathogenic microorganism, *E. coli* lipopolysaccharide as a substrate was hydrolyzed by the lipase. The isolated lipase degraded lipopolysaccharide into palmitic acid (Fig. 5). The lipase had an ability to hydrolyze *E.coli* lipopolysaccharide, suggesting that the oat lipase may possess physiological role to defend against pathogens.

Discussion

Most attention has been paid to the role of plant lipase TAG hydrolase, which hydrolyzes TAG in seeds and provides energy in the early germination stage. However, it has recently been found that plants lipases play a variety of roles. Bacterial GDSL family lipase (family II) were identified from *Aeromonas sp*. for the first time (Volokita et al., 2011). Since then, lipase belonging to the GDSL family has been isolated from several plants including *Arabidopsis*, but researches regarding the characteristics and functions of lipase have not been fully reported.

The present study purified the lipase from the oat seedlings (Fig. 1), which showed high specific activity (Table 1). Unlike the true lipase, which has a TAG hydrolase function, the oat lipase showed high enzyme activity against fatty acids with a monoacyl chain, rather than with di- or triacyl chains, and presented higher substrate specificity against fatty acids containing short chains (C4) than long chains (C16) (Table 2). During the germination period, the lipase was expressed at high levels until 6 days after germination and then decreased drastically thereafter. Most of these lipases can be found in the upper part of the oat seedling (Fig. 2A), especially the young leaves of the upper part (Fig. 2B), and within the young leaves, the lipases are located only in the vessels and sieve tubes (Fig. 3).

Among bacterial leaf blights, the *P. syringae* pv. *oryzae*. bacteria are limited to the leaf vein and cause bacterial blight

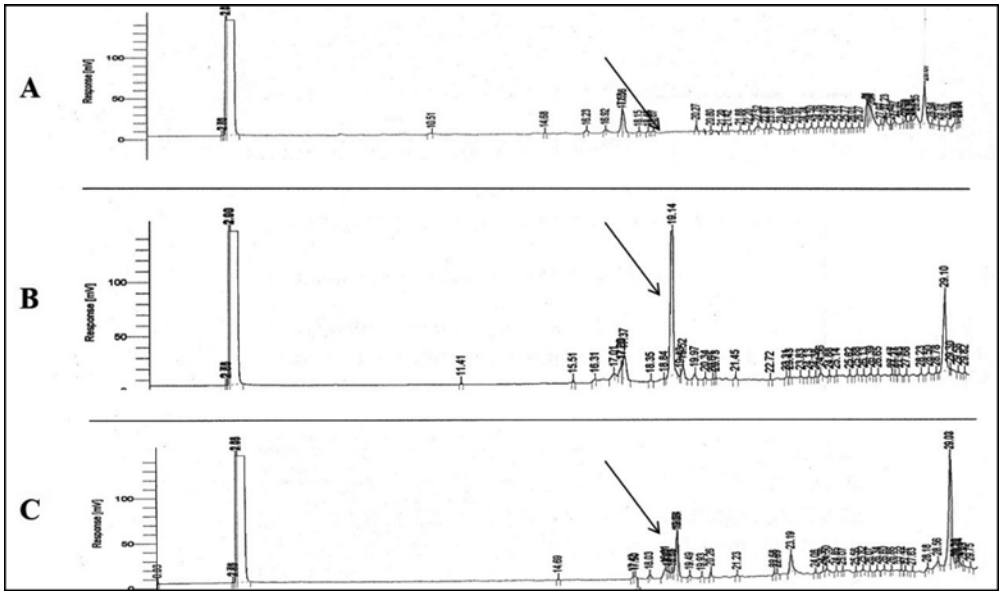

Fig 5 GC analysis of lipase hydrolyzate of lipopolysaccharide from *E. coli.* Conditions: GC-FID (Agilent GC- 6890, USA), the flow rate of carrier gas (He) was set at 2.5 mL/min. The oven temperature was programmed to initiate at 160°C and was held for 2 min. The temperature was raised to 200°C at a rate of 20°C/min and held for 4 min, and finally increased to 270°C at a rate of 5°C and held for 23 min. Column: SPD-1 fused-silica capillary column (30 m×0.53 mm I.D., 0.10 m film thickness. A: lipopolysaccharide only, B: palmitate standard. C: lipopolysaccharide hydrolyzate.

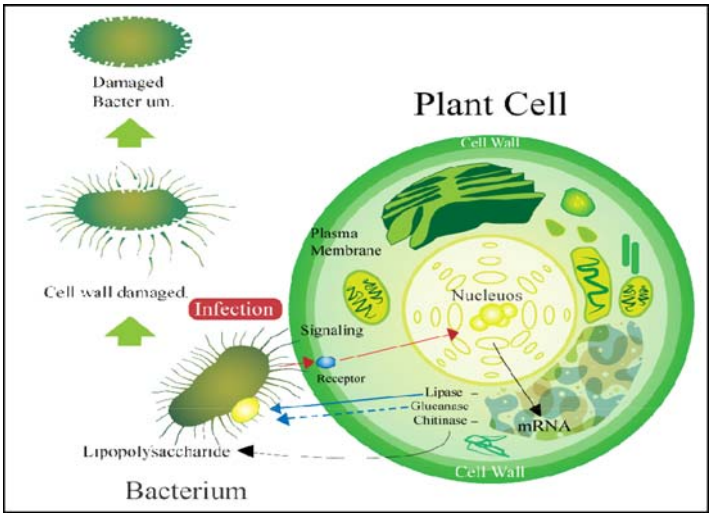

Fig. 6 A proposed function of oat lipase: Infection of bacteria signals to the nucleus of the plant, where it induces expression of enzymes involved in hydrolysis of the bacterial cell wall, such as lipase and glucanase.

disease. Because the distribution of lipase appears to be limited to the vessel and sieve tube, the physiological function of lipase involved in pathogen resistibility was investigated by infecting with *P. syringae* pv. *oryzae*. The results indicated that these bacteria induced biosynthesis of lipase (Fig. 4).

Due to these inherent characteristics, the oat lipase is clearly distinguished from the attributes of the true lipase. In other words, the oat lipase has different substrate specificity than the true lipase, and it also has activity that hydrolyzes the cell walls of bacteria (Fig. 5). The level of enzyme increased when treated with the pathogen *P. syringae* pv. *Oryzae*, which was infected in the vessels and sieve tubes and caused plant blight disease, and the oat lipase also appeared at high levels in the vessels and sieve tubes of young oat leaves. A previous study found that lipases that have similarities with the family I lipase (TAG lipase) and GDSL family lipase are involved in the defense mechanism associated with microorganism infection (Kwon et al., 2009). Several well-known lipases have been reported to be involved in the defense mechanism against microorganism infection. The oat lipase of the present study is also considered to have a defensive function

against microorganisms such as *Pseudomonas syringae* pv. *oryzae.* In other words, once *P. syringae* pv. *Oryzae* is infected, the expression of lipase will increase. The lipase also exists in most vessels and sieve tubes, where the microorganisms inhabit. In addition, the lipase has a function to decompose lipopolysaccharide, presenting a pathogen resistance defense mechanism of the lipase (Fig. 6). When pathogens attack through the vessel and sieve tube and they are recognized by the receptors located in the cell membranes that constitute the vessel and sieve tube, the signal is delivered to the nucleus and then the mRNA of defense mechanism-related proteins, such as, lipase, glucanase, and chitinase, will be expressed. Expression of these proteins will hydrolyze the cell membranes of microorganisms. This mechanism will loosen the cell membranes of microorganisms and consequently result in microbial cell death. Fig. 6 showed that lipase and glucanase hydrolyze the lipopolysaccharide of bacteria.

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