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Use of natural fluorescent triacyglycerols from *Parinari glaberrimum* to detect low lipase activity in *Brassica napus* seedlings

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Lipases (triacylglycerol acyl hydrolass EC 3.1.1.3) are defined as enzymes that catalyse the hydrolysis of triacylglycerols, releasing long-chain fatty acids. Germinating oilseeds have been explored as a possible source of lipases for biotechnological processing of oils and fats. However, purification and sensitive assays to detect a true lipase activity in plant cellular homogenates and culture media are required. The main aim of this study was to design a convenient, specific, sensitive and continuous lipase activity assay using natural long-chain triacylglycerols (TAGs). Oil was extracted from *Parinari glaberrimum* seed kernels and the purified TAGs were used as a substrate for detecting low levels of lipase activities. An increase in the fluorescence was observed, which is due to the parinaric acid released by various lipase activities. This increase in the fluorescence intensity is linear with time and proportional to the amount of lipase added. This new method, performed under non-oxidative conditions, was applied successfully to detect low lipase levels in crude protein extracts from plant seeds.

Key words: *Brassica napus*, fluorescent lipids, lipase assay, plant lipases.

INTRODUCTION

During the early steps of post germination of oil seed plants such as Brassica, sunflower or castor bean, growth depends on the carbon resources stored as triacylglycerol. Triacylglycerol are stored in the seeds as oleosomes or lipid bodies. They are hydrolyzed by a lipase, generating fatty acids that are metabolized in the peroxysomes to yield acetyl-CoA. Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are defined as the enzymes which catalyze the hydrolysis of storage triacylglycerols, releasing long-chain fatty acids (Brockerhoff and Jensen, 1974; Brune and Goitz, 1992; Jaeger et al., 1994). These enzymes play a capital role, particularly in oilseeds, in which storage lipids provide the respiratory fuel for seedling growth. When compared with animal, bacterial and fungal lipases, little is known about plant lipases.

Interest in lipases from different sources (animal, micro-organisms and plants) has markedly increased in the last decade due to the potential industrial and technical applications. Lipases have been found to be implicated in the oil and fat bioconversion (esterification, lipolysis and transesterification reactions) of triacylglycerols and other lipids in the presence of only small amounts of water as well as hydrolysis in aqueous media (Hassanien and Mukherjee, 1986). One common feature of all the plant lipases is that they are present in very small amount in biological samples such as in plant seeds and the low

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level of the lipase activity. Their activity can not easily be detected using titrimetric methods, applied for animal and fungi lipases. To detect plant lipase activity, for purification and food quality analysis purposes, more sensitive methods are required. Methods using radiolabeled substrates (Belfrage and Vaughan, 1969; Briquet-Laugier et al., 1999) are very sensitive but cannot be monitored continuously. Moreover, colorimetric methods (Lin and Huang, 1983) need time-consuming organic solvent separation steps to isolate the released fatty acids and they cannot be used continuously. An easy and continuous assay, compatible with low lipase levels have been developed in the LLE- Marseille (Beisson et al., 1999), using fluorescent TAGs extracted from *Parinari glaberrimum* seeds. In the present work, we have used the, fluorimetric method, in order to compare it to the other lipase activity tests. We have tested it using the supernatant S10-0day, S10- 3days and the immunopurified rapeseed lipase (IRSL).

MATERIALS AND METHODS

Lipase source

A supernatant solution (S10) obtained by centrifugation at 10,000 x *g* for 30 min of the crude extract of *Brassica napus* seedlings, the immunopurified rapeseed lipase (IRSL) (Belguith et al., 2009) and *Humicola lanuginose* lipase (HLL) were used. Control experiments were carried out by using lipase samples heated at 95°C for 5 min.

Lipase activity assays

Lipase activity was determined by measuring fatty acids produced by triacylglycerol hydrolysis using the colorimetric method (Lin and Huang, 1983), a radioactive method and a fluorimetric method developed by the laboratory of Professor Verger (Beisson et al., 1999). All experiments were carried out in triplicate.

The colorimetric method

Lipase activity was determined by measuring free fatty acids produced by triacylglycerol (TAGs) hydrolysis using a colorimetric method (Duncombe, 1962). Experiments were carried out in a Teflon screw-top glass test tube, in a total volume of 1 ml. The reaction mixture contained 50 mM triolein emulsified in 5% (w/v) arabic gum, and 1 M Tris-HCl at pH 7.5 (Lin and Huang, 1983). Reactions were started by addition of 50 µl enzymatic solution and allowed to proceed for 20 min in a shaking bath at 30°C. Appropriate controls were included and reactions were stopped with 5 ml of cold chloroform. Fatty acids released were extracted and converted to copper soap using 0.1% (w/v) sodium diethyl dithiocarbamate. The copper complex was subsequently estimated spectrophotometrically at 440 nm.

The radioactive method

In radiometric assays, lipase activity was measured using TAGs containing radiolabelled acyl chains (243 cpm/ml) as a substrate (Beisson et al., 1999). 20 ml of the rapeseed lipase preparation was incubated at pH 7.5 in the presence of 10 ml of radiolabeled triolein (22 Ci/mM) (Perkin-Elmer), 1% sodium taurodeoxycholate (NaTDC) and 7.6 ml of 4 M $CaCl_2$, in a final volume of 200 ml. After each 15 min, 50 ml of the reaction mixture were added to 1 ml of the stopping buffer. Then, a mixture of methanol/chloroform/heptanes (21:18:15; v/v/v) was added to extract the radiolabeled free fatty acids. After a centrifugation at 13,000 *g* for 2 min, 200 µl of the aqueous upper phase were taken and mixed with 8 ml of scintillation liquid (Hionic FluorTM Packard BioScience B.V.). The radioactivity of the tritium result of the hydrolysis of radiolabeled TAGs was counted on a Beckman LS 1801 apparatus.

The fluorimetric method

Preparation of the fluorescent TAG solution

Fluorescent TAGs were extracted from the seed kernels of *Parinari glaberrimum*. A stabilized crude lipidic extract from seed of *P. glaberrimum* is commercially available from Molecular Probes, Inc., and stored in the dark at -20°C under an argon atmosphere. 50 ml of the crude lipidic extract was dissolved in 1 ml of diethylether containing 0.01% (w/v) butylhydroxytoluene (BHT) as antioxidant. The TAGs were isolated by preparative TLC under an argon atmosphere and the purity was checked by TLC. Purified TAGs were stored in an ethanol solution in the presence of 0.01% (w/v) BHT (stock solution), under conditions identical to those for the crude lipidic extract. The stock solution concentration was determined by measuring the dry weight after evaporating ethanol under a stream of nitrogen and found to be 1.8 mg/ml (Beisson et al., 1999).

Preparation of the emulsions

After evaporating ethanol under a nitrogen stream, 3 mg of purified TAGs was placed in a 0.5-ml polypropylene microtube, and 100 µl of the following buffer was added: 50 mM Tris-HC1 (pH 8) containing 3% gum arabic, 4 mM NaTDC, 100 mM NaC1, 6 mM $CaCl_2$ and 0.001% (w/y) BHT. The microtube was kept closed under argon and the mixture was sonicated for 30 s in a sonicating bath (35 kHz and 30 W) (Beisson et al., 1999).

Parinaric acid-based lipase assays

The buffer (990 µl) and the TAG stock solution (10 µl) were added consecutively to a quartz cuvette of 1.5 ml (optic path-length 1 cm) containing a magnetic stirrer 8 mm in length. The final TAGs concentration was 18 µg /ml. The above mixture was slightly turbid and requires continuous stirring to ensure homogeneity. The cuvette was kept under nitrogen using a Teflon cap. After gently shaking the cuvette, it was left to equilibrate at 25°C. Lipase solution was injected and the fluorescence was read at regular intervals under continuous stirring in a spectrofluorimeter (SFM 25 from Kontron). Excitation was at 324 nm and emission was at 420 nm. The following standard buffer (pH 8) was used: 4 mM NaDTC, 100 mM NaCl, (6 mM $CaCl_2$, 0.001% (w/v) BHT) (Beisson et al., 1999). All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

More than half of the fatty acids extracted from *Parinari glabemmunm* oil are known to be 9, l1, 13, 15-octadecatet-raenoic acid (Riley, 1950), which is the cis, trans, trans, cis isomer: α-parinaric acid or cis-parinaric

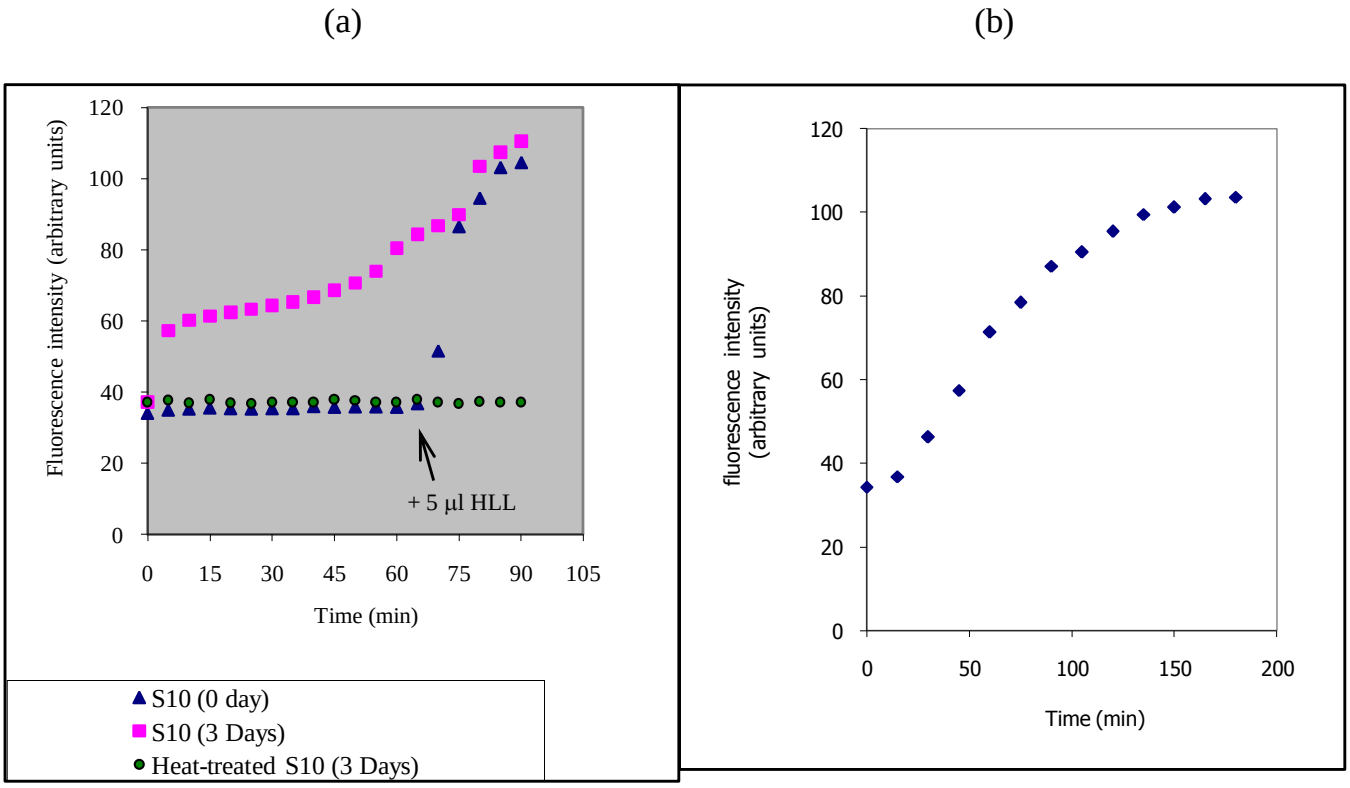

Figure 1. Kinetics of hydrolysis of natural fluorescent TAGs from *P.glaberrimum*. (a): upon incubation with 100 µl of rapeseed lipase supernatant from dry seeds (S_{10}-0day) and the 3-day-old germinating rapeseeds supernatant. 5 µl of HLL was added to the S_{10}-0day cuvette after 60 min; (b) upon incubation with 20 µl of IRS-lipase. Lipase solution was injected in l ml of the standard reaction medium (pH 8), containing 16 µg of fluorescent TAGs, 50 mM Tris, 100 mM NaTDC, 6 mM $CaCl_2$ and 0.001% BTH. Fluorescence was read at regular intervals under continuous stirring in a spectrofluorimeter (SFM 25 from Kontron). Excitation was at 324 nm and emission at 420 nm.

acid (Riley, 1950; Gunstone and Subbarao, 1967).

We determined a UV absorption spectrum of the TAC stock solution diluted 100-fold in ethanol (data not shown), which gave two peaks at 304 and 319 nm, as previously reported to occur in the case of pure n-parinaric in ethanol (Sklar et al., 1977). Comparisons between the unique spectroscopic characteristics of pure parinaric and the absorption spectrum of our purified TAC confirmed that this fluorescent fatty acid was indeed present in the TAC; stock solution.

The excitation spectrum of *P. glaberrium* TAGs was dispersed in the standard buffer (pH 8). At the maximum excitation wavelength (324 nm), the emission spectrum displayed a broad bell-shaped curve centred around 420 nm (Belguith et al., 2009).

We investigated the change in the fluorescence intensity during the course of *P. glaberrium* TAGs lipolysis, in the presence of 100 µl of the supernatant obtained from dry seeds (S_{10}-0day) and a 3-day-old germinating rapeseeds supernatant (S_{10}-3days).

The fluorescence was recorded versus time; 10 min and 180 min. Figure 1a shows that in the presence of dry seeds supernatant S_{10}-0day, no change in the fluorescence intensity was detected. After 65 min of incubation, we injected 5 µl of *Humicola lanuginose* lipase (HLL) in the tested supernatant S_{10}-0day cuvette assay; we observed that the fluorescence at 420 nm increased sharply. Our result suggests the absence of lipase activity in dry rapeseeds. It seems that lipase was virtually absent in dried seeds and synthesized *de novo* during the stage of germination, as described in corn seeds (Huang et al., 1987) and castor bean seeds (Maeshima and Beevers, 1985).

Using 3-day-old germinating rapeseeds supernatant, a significant level of lipase activity was detected after a few minutes of incubation. The increase in the fluorescence intensity was linear with time during at least 90 min of incubation, with a specific activity of about 92 $nkat.mg^{-1}$. Furthermore, no change in fluorescence intensity was detected when heat-treated S_{10}-3day was used (Figure 1a). It seems that the rapeseed lipase is a heat-labile enzyme.

We tested the fluorimetric method, using the immuno-purified rapeseed lipase (Figure 1b). The fluorescence was recorded versus time; 10 min and 180 min. A significant level of lipase activity was detected using 20 µl of

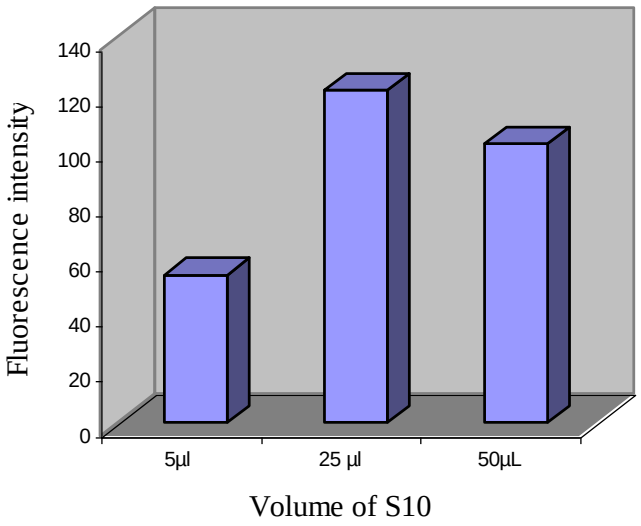

Figure 2. Effect of variation of the amount of S10 on lipase activity. Fluorescence intensity was measured after 60 mn of incubation.

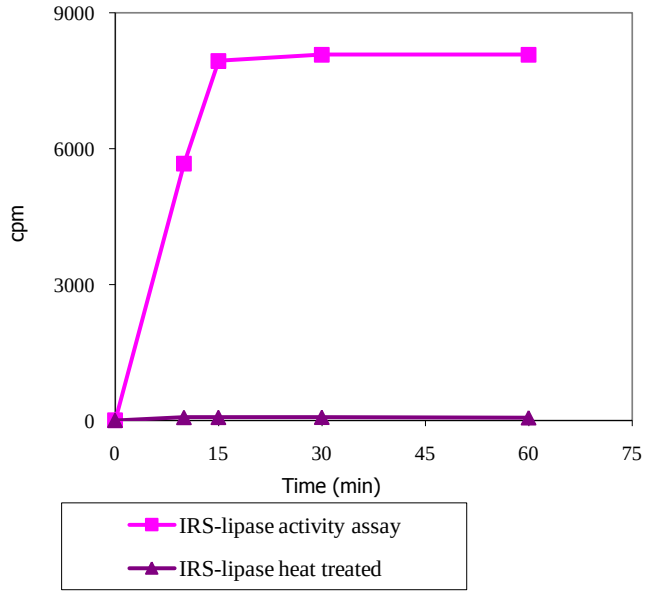

Figure 3. Lipolytic activity on radio-labeled triolein substrate. Test of the IRSL activity, using 20 µl of native and heat treated ISRL incubated at pH 7.5 and 37°C, in the presence of 10 µl of radiolabeled triolein.

rapeseed lipase. Figure 1b shows that the increase of relative fluorescence was linear with time during 120 min of incubation, with a specific activity of bout 92 nkat.mg^{-1} and after that, it reached a plateau. Moreover, we studied the variation of fluorescence intensity, using 5, 25 and 50 µl of the 3-day-old germinating rapeseeds supernatant. Figure 2 shows that in the presence of 25 µl, we had the higher lipase activity level. This result suggests that the increase in the amount of protein, induce a reduction of the lipolysis. This phenomenon seems to be due to the presence of molecules in S_{10} inducing a steric hindrance, a diminution of the interfacial tension (lipid/water) or the presence of a non specific inhibitor in the crude extracts.

Our results demonstrate that the rapeseed lipase hydrolyse efficiently the *P. glaberrimum* TAGs and confirm that these natural TAGs are therefore specific, convenient and sensitive lipase substrate.

In order to compare the obtained results in the presence of the IPRL, by the fluorimetric method, we used the radioactive method. Lipase activity was measured using TAGs containing radiolabelled acyl chains (243 cpm/µl) as a substrate. 20 µl of the IRSL were incubated at pH 7.5 in the presence of 10 µl radiolabeled triolein (243.092 cpm/µl), 1% NaTDC, 7.6 µl 4 M of $CaCl_2$, in a final volume of 200 µl. We quantify the cpm result of the hydrolysis of radiolabeled TAGs.

Figure 3 shows a linear kinetic for 15 min, after that, it reached a plateau in the presence of IPRL. No lipase activity was observed in the heat-treated IRSL for 5 min at 90°C used as a control test; our finding suggests that the IRSL is a heat-labile enzyme. Our results demonstrate that the IRSL presents a high activity level with a specific activity of about 94 nkat.mg^{-1}, using the radioactive method. These data are very close to the obtained ones using the fluorimetric method.

The tetrahydrolipstain is considered as the first irreversible and selective inhibitor of lipases (Hardvary et al., 1990). These authors had demonstrated that the THL bind specifically to the serine- residue of the catalytic site. We had tested the THL effect on rapeseed lipase activity using the fluorimetric method. Two cuvette assays were prepared; in the first, we incubated the rapeseed lipase alone as a positive control and in the second, we added 5 µl of THL after 20 min of incubation. We observed (Figure 4) that the increase of the fluorescence intensity measured at 420 nm can be stopped readily by the addition of THL and it reached a plateau at about 70 (arbitrary units). This inhibition by THL suggests the presence of a serine-residue in the catalytic site of rapeseed lipase and that serine triacylglycerol acyl hydrolase is the major lipases.

The present work documents the high sensitivity of the fluorimetric method related to the presence of *P. glaberrimum* TAGs of naturally conjugated polyene fatty acid which does not contain any chemical substituents likely to create steric hindrance. The tested fluorimetric method is based on the use of innovative substrates which are more specific and/or sensitive as compared to many of the methods currently in use for the lipase activity tests.

However, this continuous method proposed here differs from the previous methods in that; it measures the hydrolysis rate of natural fluorescent TAGs under non-oxidative conditions. It can be applied to measure true lipase activities and to detect low lipase levels such as in plant crude seed extracts.

In conclusion, while there is no doubt that the fluorimetric

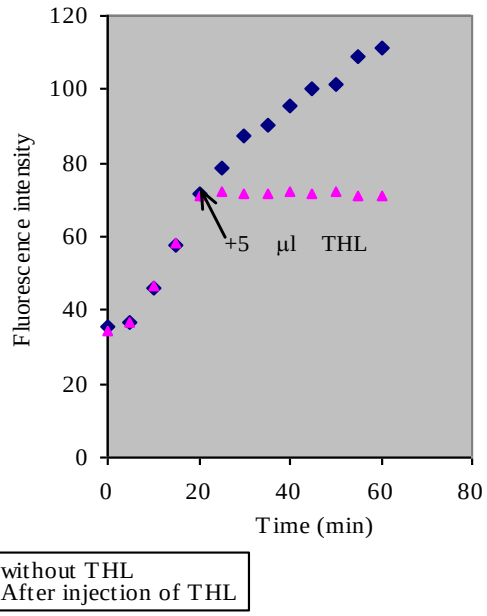

Figure 4. Kinetics of hydrolysis of naturally fluorescent TAGs from *P. glaberrimum* upon incubation with 20 µl of rapeseed lipase incubated in 1 ml of the standard reaction medium (pH 8), containing 16 µg of fluorescent TAGs, 50 mM Tris, 100 mM NaTDC, 6 mM CaCl and 0.001% BTH. In a second cuvette assay, 5 µl of THL was added after 20 min of incubation.

method assays provide interesting data to examine hydrolysis within aqueous systems, the *P. glaberrimum* TAGs lipase assay appears to be more reproducible, sensitive, rapid and specific for true lipases.

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