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Plant Physiology and Biochemistry 44 (2006) 611–617
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Research article

Assaying lipase activity from oil palm fruit (*Elaeis guineensis* Jacq.) mesocarp

G.F. Ngando Ebongue[a,*], R. Dhouib[c], F. Carrière[c], P.-H. Amvam Zollo[b], V. Arondel[c]

[a] *Centre spécialisé de recherche sur le palmier à huile (CEREPAH) de La Dibamba, BP 1001, Douala, Cameroon*
[b] *Department of Biochemistry, Faculty of Science, University of Yaoundé-I, BP 812, Yaoundé, Cameroon*
[c] *Laboratoire d'enzymologie interfaciale et physiologie de la Lipolyse (EIPL), UPR 9025, IBSM-CNRS–université de la méditerranée, 31, chemin Joseph-Aiguier, 13402 Marseille, France*

Received 6 July 2006; accepted 12 September 2006
Available online 29 September 2006

Abstract

The mesocarp of mature oil palm fruit undergoes intensive triglycerides hydrolysis upon abscission and bruising. This generates such a high amount of free fatty acids that the oil might become unfit for human consumption without appropriate refining. The lipase (EC 3.1.1.3) involved in the breakdown of the oil is not stable after homogenization of the tissue in aqueous buffers. In this study, we have devised a solvent-based procedure that allowed us to obtain fractions with stable lipase activity. Using these fractions, we have determined the optimal conditions for assaying mesocarp lipase activity. The activity was highest at a temperature of 35 °C and a pH of 9. The lipase was found to be strictly calcium dependent. The specific activity of the lipase measured in optimal conditions was found to be 33 μmol fatty acids released min^{-1} mg^{-1} protein using olive oil as substrate. The mesocarp contains about 190 U of lipase g^{-1} fresh weight. This activity was found to be inhibited by the lipase inhibitor tetrahydrolipstatin (THL), suggesting that the lipase is a serine hydrolase.
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Keywords: Lipase; Oil palm; Fruit mesocarp; *Elaeis guineensis*; Oil acidity; Free fatty acids

1. Introduction

Oil palm tree is the world most important oil crop, together with soybean, with more than 30 millions tons of oil produced in 2004 [18]. The oil can represent up to 80% of the dry weight of the mesocarp. Mesocarp lipase (EC 3.1.1.3) is one of the lipid-related enzymes of palm which has been the most studied, because of its strong negative impact on oil quality [23]. The enzyme is activated upon abscission of the fruit and also when the fruit is bruised. According to Desassis [6], 15 min are enough to hydrolyze 40% of the oil of a bruised fruit. The fruits need to be heated immediately after harvest to inactivate the lipase and to prevent an unacceptable level of free fatty acids in the oil. Initial studies had concluded to the absence of an endogenous lipase in palm fruit mesocarp [19,28]. It was supposed that triacylglycerol (TAG) hydrolysis was due to exogenous lipases secreted by fungi [12]. Since then, three groups have shown independently that a lipase does indeed exist in the fruit [1,10,21]. The authors have fractionated the tissues by centrifugation and/or filtration and have found that lipase activity is exclusively associated to oil-bodies. This has been confirmed by a histo-chemical study [17]. According to Sambanthamurthi et al. [20], the in situ lipase activity of the fruit is strongly stimulated by low temperatures. This property has been used later by the same authors as a substitute to a direct enzyme assay to quantify lipase activity from different genotypes [22]. However, Henderson and Osborne [10] reported that cold-exposed fruits yielded extracts which lipase activity was strongly reduced. There are other conflicting data. According to Abigor et al. [1], the optimal pH of activity is 4.5 while others reported a neutral [10] to basic [21] pH optimum. Similarly, the same authors reported significantly different optimal temperatures. Also, Henderson and Osborne [10] pointed out the lack of stability of the enzyme.

Assaying properly lipase activity is not an easy task, because of the non-solubility of the substrate and the strong dependence of the activity on the physico-chemical state of

Abbreviations: EDTA, ethylene diamine tetra-acetate; GA, gum Arabic; TAG, triacylglycerol; THL, tetrahydrolipstatin.

* Corresponding author.
E-mail address: caiman2307@yahoo.com (G.F. Ngando Ebongue).

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doi:10.1016/j.plaphy.2006.09.006

the substrate [3]. In the case of palm oil lipase, the task is further complicated by the non-stability of the enzyme. Henderson and Osborne [10] did assay lipase activity in mesocarp powder (or oil-bodies), that is using a fraction that contains both the enzyme and the substrate. They added trace amounts of emulsified radioactive triolein to record the release of fatty acids by scintillation counting. In these conditions, it is not possible to estimate a specific activity because the true specific radioactivity of the substrate is unknown. Another difficulty is the availability of fresh biological material. The extract needs to be prepared and assayed for lipase activity as soon as possible after harvest. The fruits need to be mature but not bruised. Also, they must not be exposed to low temperatures. It is only by controlling those parameters that it will be possible to quantify reproducibly lipase activity. This absolute quantification is essential if one wants to select low lipase genotypes for subsequent breeding. Therefore, we have reinvestigated the conditions of measure of palm lipase in order to elaborate an easy, quantitative and repeatable method for assessing endogenous lipase activity in the oil palm mesocarp.

2. Results

2.1. Enzyme activity of the aqueous oil palm mesocarp extract

Lipase activity of mesocarp samples was assayed on fresh fruits about 24 h after harvest. The mesocarp was quickly homogenized in 10 vol of 100 mM BisTris (pH 7) and assayed as such for lipase activity on olive oil. An activity of 190 ± 17 U g^{-1} of fresh mesocarp was recorded. Extracts prepared similarly from frozen fruits yielded about the same activity. The activity was found to be labile, whether the extract was made from fresh or frozen fruits. About 30% of the activity was lost after an hour and 90% after 24 hours. Keeping the extract on ice, adding protease inhibitors or cysteine had no effect on the stability of the activity (data not shown). Also, the enzyme was found to be unstable during the assay as the kinetics was linear for less than 1 min. The rate of hydrolysis at 5 min was about 20% lower than the rate at 1 min using optimal assay conditions (see Section 2.7).

After centrifugation of the homogenate, no lipase activity could be detected either in the aqueous phase or in the pellet. All the activity was found in the oil-body fraction.

2.2. Preparation of a stable extract

Lyophilized mesocarp slices were found to keep a stable lipase activity when stored for several months at 4 °C. Samples were prepared by grinding the lyophilized mesocarp in a mortar down to a fatty paste. A lipase activity of about 20 U g^{-1} of paste could be detected. This represents about 10% of the activity recorded from a crude aqueous homogenate (see above). This is probably due to the fact that most of the enzyme in the fatty paste was not accessible to the substrate during the assay. The fatty paste was fully delipidated using several solvent mixtures. This led to a complete loss of lipase as no activity could be detected either in the residual delipidated powder or in the solvent washes.

A partial delipidation was carried out by homogenizing lyophilized mesocarp in hexane as described in Section 5. A strong lipase activity (250 ± 14 U g^{-1} dry mesocarp on olive oil) could be detected. When expressed on a fresh fruit basis, the value is about 200 U g^{-1}, which is comparable to the value obtained using a crude aqueous homogenate. Lipase activity was found to associate exclusively with particles not solubilized by hexane. Further delipidation with hexane led to a 30–50% loss of activity.

The partially delipidated extract was found to contain still 20–25 mg lipids when starting from 100 mg lyophilized mesocarp. It was stored as such (that is in hexane). The lipase activity remained stable for at least 4 days at 4 °C and for more than 5 weeks at –20 °C. This partially delipidated mesocarp extract was used for further analysis.

2.3. Effect of pH

When using olive oil as substrate, the activity was tested between pH 6.5 and 12.0 (Fig. 1). The enzyme is not active below pH 7.0 and above pH 11.5. The optimal activity ranges from pH 9 to 10. However, at pHs above 9.0, the kinetics is linear for 1–2 min only, against 5 min at least when the pH is equal to or below 9.0. This suggests that the enzyme is less stable at high pHs. Therefore, we chose to assay it at pH 9.0. The activity on olive oil was also assayed at lower pHs by back titration. No significant activity could be detected between pH 4 and 6.

Assays were also carried out using tributyrin and trioctanoin as substrates. The enzyme was tested between pH 4.5 and 9.0 (data not shown) and the maximal activity was recorded at pH

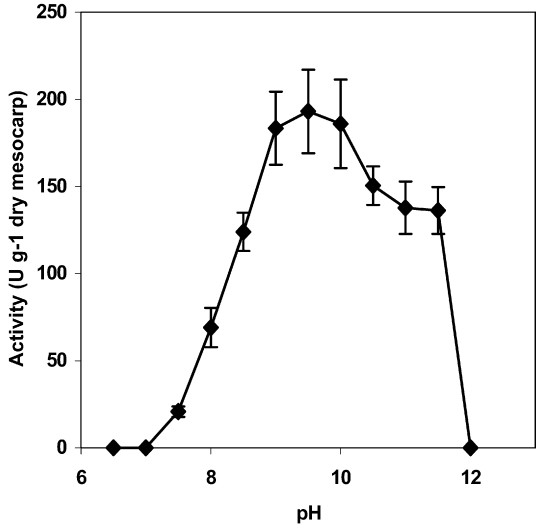

Fig. 1. Lipase activity as a function of pH. The reaction mixture contained 150 mM NaCl, 2 mM Tris–HCl, 10 mM $CaCl_2$ and 10 ml of a 10% (v/v) olive oil/GA solution (1%, w/v) in a final volume of 30 ml. Assays were carried out at 35 °C.
Values are means of three independent assays ± S.E.

7.0 (see Section 2.5). The enzyme extract was totally inactive on these substrates at pH values below 5.

2.4. Effect of temperature

The partially delipidated mesocarp extract was active at temperatures ranging from 20 to 50 °C (Fig. 2). Maximal activity was recorded at 45 °C using olive oil as substrate. The kinetics was linear for 5 min at least when the activity was assayed below 35 °C. Above this value, the activity declined after 1 or 2 min, suggesting that the enzyme is less stable at high temperatures. Therefore, we chose 35 °C to assay the activity.

2.5. Substrates hydrolyzed

Maximum activity of the partially delipidated extract was recorded with tributyrin and trioctanoin as substrate (706 ± 31 U g^{-1} and 816 ± 59 U g^{-1} dry mesocarp, respectively, at pH 7), (Fig. 3). The activity on olive oil was 250 ± 14 U g^{-1} in optimal conditions (see Section 2.7) and 270 ± 10 U g^{-1} on palm oil. The extract was also capable of hydrolyzing phosphatidylcholine (115 ± 4 U g^{-1}) but not cholesteryl oleate.

2.6. Effect of calcium and other compounds

Lipase activity was found to be fully inhibited by 1 mM ethylene diamine tetra-acetate (EDTA). The activity could be restored with $CaCl_2$ but not with $MgCl_2$ (Table 1). The optimal concentration of $CaCl_2$ was 10–50 mM (Fig. 4). NaCl did not exhibit any significant effect at concentrations ranging from 0 to 150 mM. It had a slight inhibitory effect (about 20% inhibition) at 450 mM. Sodium taurodeoxycholate shows a slight positive effect on the activity below 0.5 mM and a slight negative effect above 1 mM. The concentration of gum Arabic (GA) was found to affect the activity, depending on the pH. At pH 7, the lipase is not active at a GA concentration of 0.33% while it exhibits an activity of 50 U g^{-1} dry mesocarp at a 3.3% GA. At pH 9, 3.3% GA inhibits the activity by about 45%. GA had already been reported to be tensio-active and to affect lipase activity [27]. Tetrahydrolipstatin (THL) reacts with the active site serine and inhibits lipases [9]. Upon reaction with the inhibitor, a covalent bond is formed with the catalytically active serine residue. Our results indicate that about

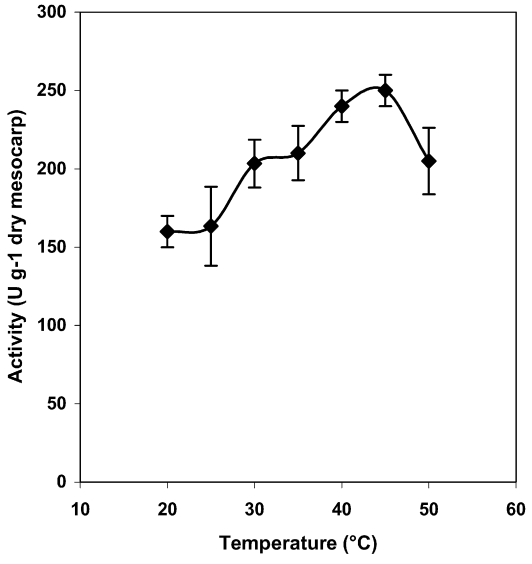

Fig. 2. Lipase activity as a function of temperature. The reaction mixture was the same as for Fig. 1. Assays were carried out at pH 9.
Values are means of three independent assays ± S.E.

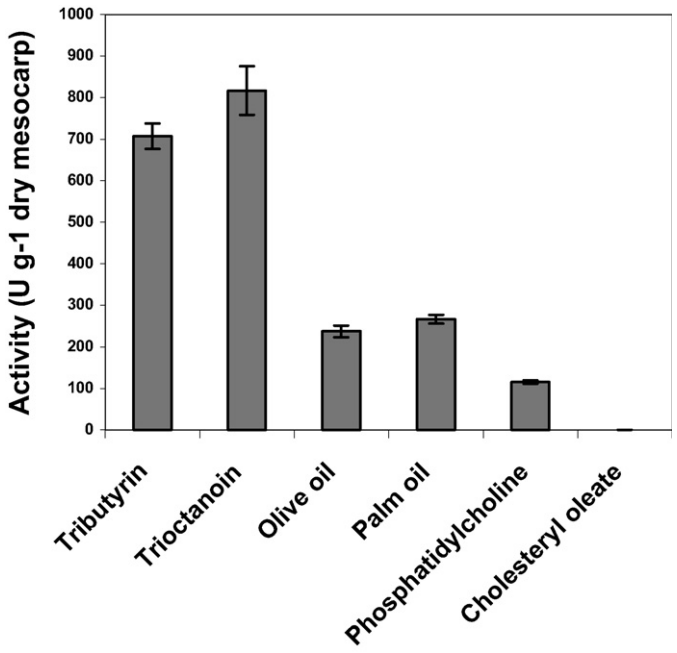

Fig. 3. Hydrolysis of different substrates. Olive oil or palm oil was emulsified in a 1% (w/v) GA solution. Assays were carried out in 150 mM NaCl, 2 mM Tris–HCl, 10 mM $CaCl_2$ at 35 °C, pH 9 for palm oil and olive oil (pH 8 for tributyrin and trioctanoin).
Values are means of three independent assays ± S.E.

Table 1
Effect of some components on lipase activity

Substrate	Changes to reaction mixture	Activity ($P = 100\%$)
Olive oil	None	100
	3.3% (w/v) GA	43
	EDTA 3 mM (minus $CaCl_2$)	0
	NaCl 450 mM	80
	$MgCl_2$ 10 mM	60
	Na taurodeoxycholate 0.1 mM	119
	Na taurodeoxycholate 1 mM	71
Tributyrin	None	100
	EDTA 3 mM	0
	EDTA 3 mM + $CaCl_2$ 6 mM	52
	EDTA 3 mM + $MgCl_2$ 6 mM	0

When using olive oil as substrate, the standard reaction mixture contained NaCl 150 mM, $CaCl_2$ 10 mM, Tris–HCl 2 mM and 10 ml of a 10% (v/v) emulsion of olive oil in GA (1%, w/v) solution. Assays were carried out at pH 9, 35 °C. When using tributyrin as substrate, the experiments were carried out as follows: EDTA was introduced first. The lipase was then added and no activity was detected. $CaCl_2$ (or $MgCl_2$) was then added and the activity measured. The reaction mixture contained tributyrin (250 μl), NaCl 150 mM, Tris–HCl 2 mM and GA (0.33%, w/v). The final volume was 30 ml, the pH 7 and the temperature 35 °C.

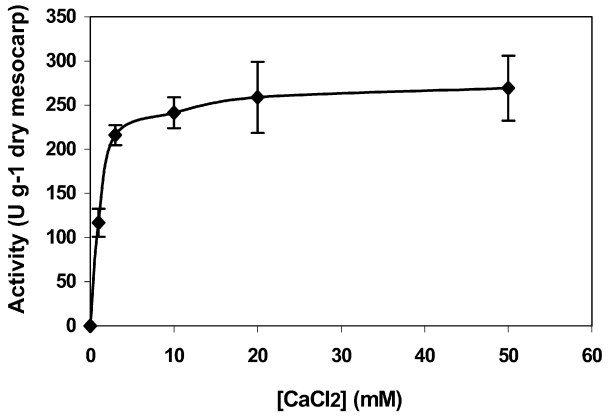

Fig. 4. Influence of $CaCl_2$ on lipase activity. The reaction mixture contained 150 mM NaCl, 2 mM Tris–HCl, 1 mM EDTA, various amounts of $CaCl_2$ and 10 ml of a 10% (v/v) olive oil/GA solution (1%, w/v) in a final volume of 30 ml. Assays were carried out at pH 8.5, 35 °C.
Values are means of three independent assays ± S.E.

85% (i.e. 5.5 U) of the lipase activity is inhibited when pre-incubating the enzyme with 1 nmol of THL (Fig. 5).

2.7. The optimized assay

The conditions for assaying are 2 mM Tris–HCl (pH 9), 150 mM NaCl, 10 mM $CaCl_2$, 3.3% (v/v) olive oil emulsified in GA 1% (w/v) at 35 °C. The final concentration of GA in the assay is 0.33% (w/v). When the oil emulsion is prepared immediately prior to use, the amount of substrate becomes rate limiting only below a 0.33% (v/v) final concentration (Fig. 6C). The kinetics of reaction was linear for at least 5 min when using a hexane-delipidated sample (Fig. 6A). When the activity is tested using an aqueous extract, linearity is limited to less than a minute. The amounts of enzyme extract for which the rate varies proportionally range from 25 to 200 μl (i.e. 2.5–20 mg of dry mesocarp) (Fig. 6B).

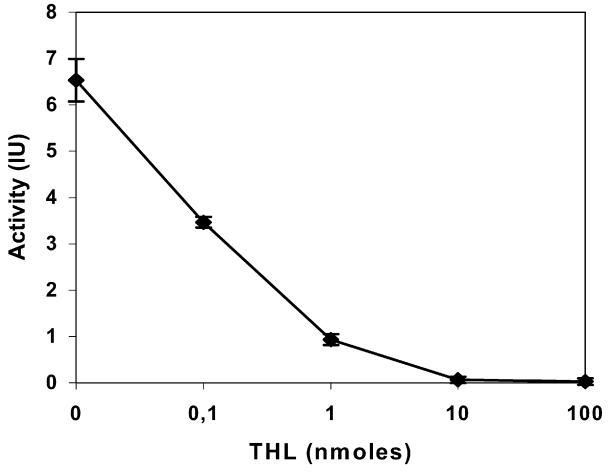

Fig. 5. Lipase activity in presence of THL. Lipase activity was assayed on enzyme extracts (7 U) pre-incubated for 1 hour with different amounts of THL. Conditions of assay are as in Fig. 1.
Values are means of three independent assays ± S.E.

Typically, 50 μl of enzyme is used per assay, which corresponds to 5 mg of dry mesocarp. This sample contains about 1 U and 1 mg of endogenous lipids.

3. Discussion

3.1. Optimization of the assay

One of the first difficulties encountered when working with oil palm lipase is the availability of appropriate biological material. The fruits need to be freshly harvested, undamaged as bruising is a strong in vivo inducer of lipase activity [6] and not exposed to chilling temperatures, which also strongly affects lipase activity [10,20,21]. Also, the fruit must not be infested by lipase-producing fungi [12,14,28]. Our results confirmed that fruits can be rapidly frozen in liquid nitrogen and kept in a freezer without affecting the lipase, as already pointed out by Henderson and Osborne [10]. Also, we showed that lyophilization fully preserves lipase activity. However, exposing the frozen or the lyophilized fruits to room temperature cause the lipase activity to decline rapidly. Also, re-hydration of lyophilized mesocarp leads to complete inactivation of the lipase.

Our results confirm that lipase activity declines rapidly in an aqueous extract [10]. Therefore, we have devised a partial delipidation procedure which allows the recovery of the same amount of activity as the aqueous extraction. However, contrarily to this procedure, partial delipidation yields a much more stable lipase, probably because of the low amount of water in the sample. Pursuing delipidation leads to a loss of activity. All activity is lost upon full delipidation. Therefore, the enzyme needs a lipid environment to remain active.

For assaying lipase, we have chosen end-point titration of free fatty acids by a pH-STAT. This technique is more appropriate than most other techniques to optimize a quantitative lipase assay [3], providing tissues are rich enough in lipase activity. GA concentration was lowered to 0.33% (w/v) in the assay, since usual concentrations (3.3%, w/v) inhibit mesocarp lipase activity at basic pH. Optimal pHs are slightly higher than those reported by Henderson and Osborne [10] and Sambanthamurthi et al. [21]. A possible reason for this is that the lipase is not stable at high pHs and that the kinetics ceased to be linear after a few minutes. This last point cannot be easily detected using the discontinuous assays described by the previous authors. Alternatively, this might be due to the high amount of tensioactive compounds (detergents, GA) used by the authors.

3.2. Biochemical properties of oil palm lipase

More than 200 lipase U g^{-1} fresh mesocarp (with olive oil as substrate) are present in the fruits that we used throughout this study. However, the mesocarp of fruits from other genotypes was found to contain more than 700 U g^{-1} fresh weight (Ngando and Dhouib, unpublished results). Calculations made from Desassis results [6] indicate that the rate of in situ lipo-

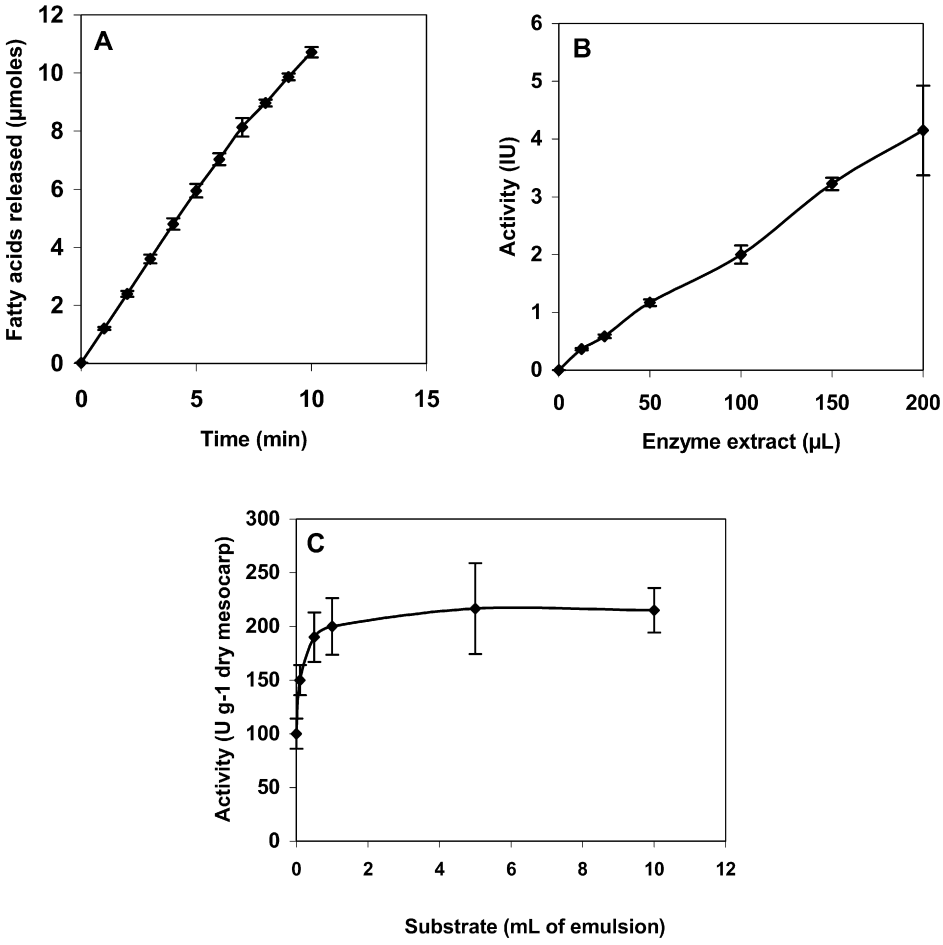

Fig. 6. Hydrolysis of olive oil TAGs as a function of time (A), amount of enzyme extract (B) and amount of substrate (C). Conditions of assay are as in Fig. 1. Values are means of three independent assays ± S.E.

lysis can be as high as 60–130 µmol fatty acids freed min^{-1} g^{-1} fresh mesocarp, which is fairly consistent with our results. This abundance makes palm mesocarp one of the plant tissues that contain the highest amount of lipase. For example, the maximal amount of lipase detected in germinating rapeseed cotyledons is about 30 U g^{-1} fresh weight, while there are about 88 ± 27 (our data) to 150 U g^{-1} (this last value is calculated from Hills and Beevers data [11]) of acid lipase in castor bean seeds. Pancreas, the richest animal tissue, contains about 7600 lipase U g^{-1} fresh weight using olive oil as substrate [29]. The specific activity of the mesocarp extract is about 30 lipase U mg^{-1} protein using olive oil as substrate. About 50% inhibition was obtained when pre-incubating the lipase with 0.1 nmol THL (Fig. 6). If one considers that all the THL molecules present in the assay are bound to the enzyme, the maximal amount of lipase is 0.1 nmol for 3 U. Therefore, the lipase has a minimal molecular turnover of 500 s^{-1} on olive oil. Assuming that the lipase molecular weight is 50,000 Da, a minimal specific activity of 650 U mg^{-1} protein can be calculated. Most true lipases have specific activities of about 1000–3000 U mg^{-1} [27]. Therefore, it is likely that the lipase represents a few percent of total mesocarp proteins. Since Sambanthamurthi et al. [21] claimed that oil-body proteins represent about 3.6% of total proteins, it is likely that the lipase is one of the most abundant oil-body protein, as it is the case for castor bean [7]. The physiological significance of this abundance remains to be understood. The enzyme is much more active on short and medium chain TAGs than on long chain TAGs, as it is the case for most lipases. The lipase activity is fully inhibited by THL, a lipase inhibitor that binds covalently to the active site serine residue. This indicates that oil palm lipase is a serine enzyme, like all known TAG lipases. Also, the extract contains an important phospholipase activity. Several TAG lipases possess a dual TAG lipase/phospholipase A1 activity [24,26]. Whether the phospholipase and the lipase activities measured are due to the same enzyme remains to be demonstrated using a pure enzyme. Interestingly, calcium is absolutely required for activity. Cytosolic phospholipases A2 have also an absolute requirement for calcium, which is not involved in the catalytic mechanism but in the interactions enzyme-lipid [15]. If calcium can stimulate the activity of most TAG lipases from mammals or micro-organisms, very few of them have been shown to be strictly calcium-dependent. The best known example is castor bean neutral lipase [11] which requires micro molar concentrations of calcium. Also, Oo [19] has shown that the lipase detected during seed germination of oil palm is calcium-dependent. This lipase is also insensitive to bile salts and has an optimal pH of 7.4.

However, it has been evidenced from an aqueous extract that excludes oil-bodies, while mesocarp lipase activity is exclusively detected in the oil-body fraction. Our results confirm the data published by Henderson and Osborne [10] and Sambanthamurthi et al. [20,21]. They strongly diverge from data by Abigor et al. [1] which concern a sulphydryl enzyme, active at acid pHs and insensitive to EDTA. We were unable to detect any lipase activity below pH 5 on short, medium and long chain TAGs.

4. Conclusions

Selecting low-lipase lines of oil palm tree is an important target for breeders. Preliminary results by Sambanthamurthi et al. [22] and us suggest that an important variability exists for this trait. Up to now, assaying lipase activity from oil palm mesocarp was not an easy task, because of the constraints on the availability and the storage of appropriate biological material and the lack of stability of the enzyme. Also, the previously published assays did not permit to obtain absolute quantification of the activity, so that it was not possible to compare results from one article to another. This is an important drawback if one wants to identify the lines with the lowest lipase activity. The optimal assay described in this paper brings up practical solutions to most of these difficulties. In addition, titration of fatty acids is a simple and inexpensive method to assay lipase activity which should be easy to implement in local palm tree breeding stations.

Our results show that lipase is very abundant in the mesocarp and represents probably a few percent of total proteins. Since it is probably one of the major protein of oil bodies as it is the case in castor bean seeds [7], a proteomic approach should allow us to identify and clone the enzyme.

5. Methods

5.1. Plant material

Fresh palm fruits (*Elaeis guineensis* Jacq. var. Tenera) used for this study were harvested at the oil palm specialized research centre (CEREPAH) of La Dibamba, Cameroon. Mature bunches (about 20 weeks after anthesis) were selected based on the abscission of less than five fruits [25]. Spikelets of fruits without any visible alteration (bruising, microbial infestation) were chosen, surface sterilized in sodium hypochlorite (0.5% active chloride) for 30 min and washed in distilled water. Fruits were stored at room temperature (20–25 °C) and never exposed to temperatures lower than 20 °C. For long term storage, fruits were quickly frozen in liquid nitrogen and stored at –80 °C.

5.2. Fractionation of mesocarp tissue

The exocarp was removed from the fruit using a scalpel blade. Mesocarp slices (2 g) were cut from the mid-region of the fruit and ground (three bursts of 30 s each at low speed) in a Waring blender with 20 ml of 100 mM BisTris (pH 7) containing, when appropriate, protease inhibitors, cystein, calcium chloride. The homogenate was centrifuged at 16,000 × *g*, 4 °C for 15 min, and three fractions obtained: a floating fatty layer on the top, a clear aqueous middle layer and a fibrous pellet. The floating fatty layer was carefully transferred with a spatula to another tube containing 20 ml of extraction buffer. The content of the tube was mixed vigorously with a vortex and centrifuged again at 16,000 × *g*, 4 °C for 15 min. The floating fatty layer was washed once more in the same conditions.

Lipase activity was assayed on the crude homogenate and the three fractions obtained after centrifugation.

5.3. Delipidation of mesocarp and preparation of anhydrous extracts

Fruit mesocarp was sliced, quickly frozen in liquid nitrogen and lyophilized. Lyophilized tissue was kept at –20 °C until use.

Total delipidation was carried out according to Verger et al. [29] using successive treatments with acetone, chloroform/butanol mixtures and diethyl oxide. Partial delipidation was performed as follows: dried mesocarp slices were ground using mortar and pestle to a fatty paste. Then, 100 mg of paste were homogenized in a micro-potter with 1 ml hexane, and the content was shaken vigorously using a vortex. The tube was centrifuged and the hexane removed without disturbing the pellet. Fresh hexane (1 ml) was added again and the pellet re-suspended by vortexing. This treatment was found to remove about 80% of the lipids. The activity was found to associate exclusively with particles not solubilized by hexane. For assaying lipase activity, the particles were re-suspended by vortexing and a sample (50 μl) was quickly pipetted using tips which end had been cut off to allow the particles to be freely taken up.

5.4. Measurement of lipase activity

Lipase activity was assayed by titrating the fatty acids released with 0.01 M NaOH using a METROHM pH-STAT. The volume of reaction was 30 ml. Stirring was adjusted so that the agitation is vigorous but the mixture does not foam. The substrate was either tributyrin (250 μl), trioctanoin (500 μl), olive oil or palm oil (1 ml). The oil was emulsified immediately before use in 1% GA (this emulsion is stable for an hour). The reaction medium contained 2 mM Tris–HCl. The effect of various salts and detergents (NaCl, $CaCl_2$, sodium taurodeoxycholate) on the activity was tested.

Phospholipase activity was assayed as described by Abousalham and Verger [2] using phosphatidylcholine as substrate at 35 °C, pH 8. Cholesteryl oleate esterase activity was assayed according to Ben Ali et al. [4].

Rapeseed lipase was assayed from a crude extract of 4-day-old seedlings according to Hoppe and Theimer [13]. Castor lipase was assayed by back titration at pH 4.5 in 150 mM NaCl, using a crude homogenate from imbibited seeds.

The pKa of oleic acid was found to be 6.7 under our optimized conditions. A correction factor was determined for pH values ranging from 6.5 to 8. At pH 8, 97% of the oleic acid is ionized. Lipase activity was expressed in international units (1 U = 1 μmol fatty acid released min^{-1}).

5.5. Lipase inhibition by THL

Inhibition experiments were carried out according to the so-called method A [8]. The enzyme extract was pre-incubated during 1 h at 4 °C with various amounts of THL solubilized in hexane. The residual lipase activity was then assayed as described above.

5.6. Protein extraction and analysis

Proteins were extracted using water-saturated phenol according to Meyer et al. [16]. Lipids were removed by extracting repeatedly the phenol phase with hexane.

The protein content was determined according to Bradford [5] using bovine serum albumin as standard.

Acknowledgements

Georges Ngando Ebongue was the recipient of a scholarship from the French embassy in Cameroon.

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