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TITRIMETRIC TEST FOR LIPASE ACTIVITY USING STABILIZED TRIOLEIN EMULSIONS

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Key Word Index—*Brassica napus*; Cruciferae; rape seed; lipase; lipolysis; pH-stat method; triolein.

Abstract—A common assay system for lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) activity is based on the measurement of free fatty acids, liberated from triacylglycerols, such as triolein, by enzymatic hydrolysis. This enzymatic reaction involves, in addition to substrate concentration, the size of the surface area of the oil-water interface in the assay system. Only sufficiently dispersed and stabilized oil-water emulsions are suitable for reproducible determination of lipase activity, e.g., in subcellular fractions of the oil storing cotyledons from rape seedlings. Stabilization of the emulsified substrate by both gum arabic (*Acacia*) and the detergent, desoxycholate, was found to be crucial to the measurement of the lipase activity in a pH-stat. Furthermore, complete ionization of free fatty acids is achieved at pH 9.0 and, consequently, highest lipase activity was found in a test medium adjusted to this pH value. Inclusion of $CaCl_2$ into the assay medium leads to a decrease in lipase activity, addition of NaCl to an increase. Preincubation of enzyme preparations with different detergents activates the lipolytic breakdown of triolein emulsions. Producing a large surface area in the triolein emulsion and stabilizing it for hours, increases the sensitivity of the titrimetric assay method. Using such conditions it was possible to determine, for the first time, the low lipase activity in non-fatty tissues, such as hypocotyls of rape seedlings.

INTRODUCTION

The enzyme lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyses the hydrolysis of various forms of fatty acyl esters and, in contrast to other esterases, needs an oil–water interface for optimum activity. Water-soluble acyl esters are negligible substrates, thus, a sharp distinction against common esterases (EC 3.1.1.1) is possible [1–3].

During germination of oily seeds, the physiological role of lipase is the breakdown of storage oils [4]. Such cellular reserve oils are stored in distinct cell compartments, called lipid bodies (oleosomes), which are stabilized by specific proteins (oleosins) to prevent coalescence [4, 5]. It is generally accepted that the site of lipase action is the surface of the lipid body [4–6]. But, for the *in vitro* determination of lipase activity there are several obstacles. For example, technical difficulties (e.g. stabilization of substrate emulsion), high activities of non-specific acylhydrolases or even intracellular inhibitors, are to be expected to affect the correct determination of lipase activity [7]. Since the site of action for lipase is the oil–water interface, an optimally large and stable surface area of lipid globules in a neutral ester emulsion is important for assaying lipase activity. For this reason, trioleoylglycerol (triolein) was found to be an ideal substrate, which can be emulsified in a hydrophilic assay system to small lipid droplets with average diameters of *ca* 3 μm [8], corresponding to an oil-in-water emulsion with an interfacial area of 750 $cm^2\ \mu l^{-1}$ [9]. It is, of course, completely water-insoluble, which is crucial to the definition of 'true' lipase activity. In the present paper, the results of studies are reported that were undertaken in order to improve the continuous titrimetric lipase assay for free fatty acids (FFA) released from stabilized triolein-water emulsions. The test method shows reliable specificity and increased sensitivity, detecting also low activities of 'true' lipase. In addition, the role of the detergent desoxycholate and various salts, in this assay, was investigated. With this optimized assay, we were able to demonstrate, for the first time, the presence of lipase activity in a non-fatty tissue, such as hypocotyls of rape seedlings.

RESULTS

Lipase activity from etiolated cotyledons of 4-day-old rape seedlings was measured, monitoring the released FFA continuously with the pH-stat technique

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using triolein emulsions as substrate, as previously described [9]. For the preparation of the reaction mixture, triolein (100 mM) dispersed in gum arabic (*Acacia*) is used and a desoxycholate (DOC) solution is subsequently added to the substrate in the reaction vessel. Employing this assay system, the apparent lipase activity decreased within 90 min from 16 to 7 nkat mg protein^{-1} (Fig. 1). When the identical enzyme preparation is assayed after storage for 5 hr in a newly prepared substrate emulsion, the initially apparent enzyme activity was measured. Again, the enzyme activity decreased over time after sonication of the oily substrate (Fig. 1). Triolein sonicated in the solution of the emulsifying agent, gum arabic, resulted in a dispersed substrate which was not suitable for the determination of lipase activity. It is likely that the surface offered to the enzyme is unstable and that the coalescence of lipid globules decreases the interface area available for enzyme action. But, when the triolein emulsion is mixed directly with the desoxycholate solution, such that a substrate emulsion containing DOC is added to the reaction vessel, a simple modification of the preparation procedure for the substrate emulsion, an apparent lipase activity of 25 nkat mg protein^{-1} was measured, which, in addition, was fairly constant for up to 10 hr storage of both the stabilized emulsion and the lipase preparation (Fig. 1).

Since with our improved test system increased apparent lipase activities were recorded, the effect of DOC on lipase activity in this assay method was investigated further. Using the same substrate concentration, identical conditions for emulsification of triolein and the same enzyme preparation, the apparent lipase activity was clearly affected by the presence of DOC in the assay system. Lipase activity determined with triolein emulsions stabilized only by the emulsifying agent, gum arabic, in the absence of DOC in the test solution, yielded an enzyme activity of 4.2 nkat mg protein^{-1}. During the period of storage of the substrate emulsion a reproducible level of lipase activity was achieved (data not shown). But, the apparent activity was more than five-fold higher when lipase was assayed with emulsions stabilized by both DOC and gum arabic. An average specific lipase activity of 23.3 nkat mg protein^{-1} was determined using such substrates. Increasing the concentrations of triolein and/or DOC in the assay medium did not improve the amounts of apparent lipase activity (data not shown).

Table 1 shows that the optimal concentration of stabilizer gum arabic results in a dramatic increase of lipase activity. With triolein dispersed in 10% gum arabic solution used in the optimized assay instead of 5%, the apparent lipase activity was found to be raised almost three-fold. Apparently, the enhanced emulsify-

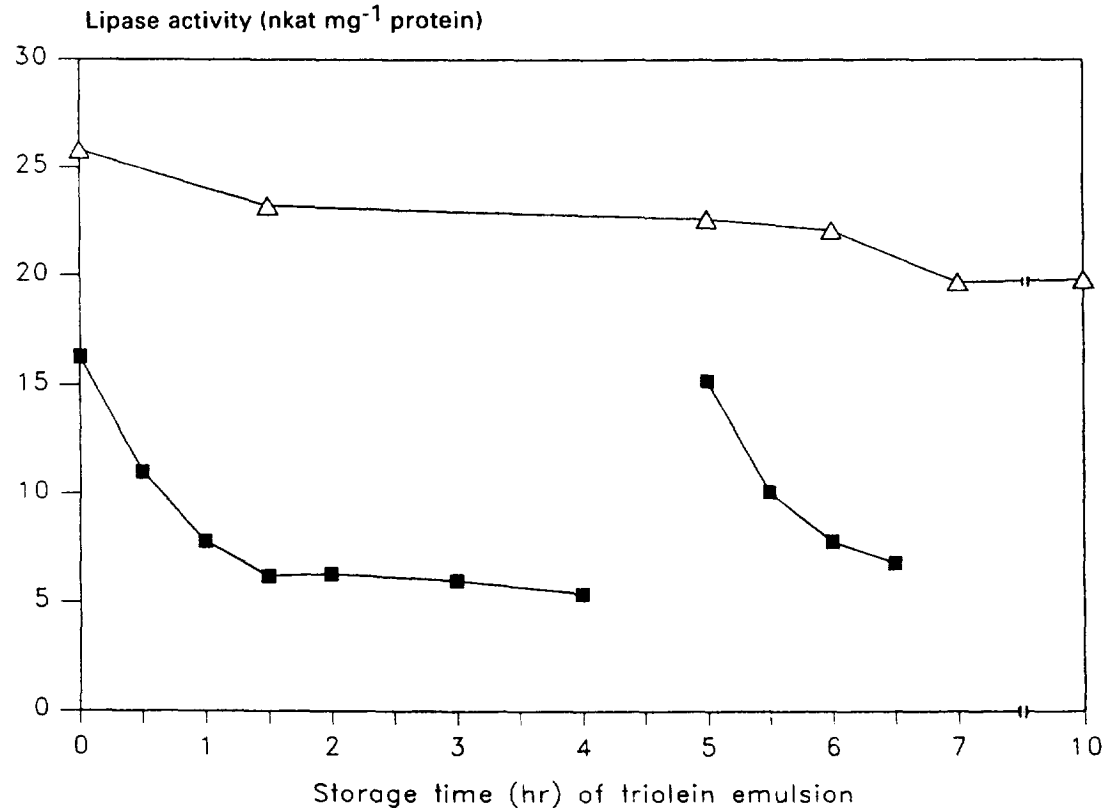

Fig. 1. Changes of lipase activity as a function of the storage time and stabilization of substrate emulsions. (■—■) Lipase activity determined with triolein emulsions stabilized by gum arabic. DOC solution was added to the substrate emulsion in the reaction vessel. (△—△) Lipase activity assayed with a triolein emulsion stabilized by both gum arabic and DOC (see text).

Table 1. Effects of concentration of gum arabic on emulsification of triolein at constant sonic energy

Gum arabic ($g\ l^{-1}$) used for emulsification of triolein	Specific activity of lipase (nkat mg protein^{-1})
50	9.3
100	27.0
150	23.9
200	24.0

ing capacity of increased gum arabic concentrations increases the surface area of the oil–water interface, and, therefore, the apparent lipase activity is at optimum, since higher concentrations of gum arabic did not increase the apparent lipase activity (Table 1).

As a second factor in the titrimetric lipase assay system, the detection and quantification of FFA depends on the ionization of FFA. The titration of oleic acids added to the reaction vessel containing DOC-stabilized emulsions or only the emulsifying agent, gum arabic, gave nearly identical results, showing that almost complete ionization of the FFA is reached at pH 9.0 (Fig. 2). Consequently, maximum activity of a lipase preparation from rape seedlings is measured by the pH-stat method at pH 9.0. However, the addition of 2.5 mM DOC into the gum arabic solution shifts the ionization of oleic acid to higher pH values (Fig. 2). At pH 9.0, only 40% of the added FFA was detected; 3.3 mM DOC in a gum arabic solution resulted in only 20% ionization at pH 9.0 (data not shown). As a consequence, the free concentration of DOC in the optimized assay, containing DOC to stabilize the emulsion, is below the critical micellar concentration of DOC (3 mM [10]), i.e., the free concentration of DOC in the assay medium does not correspond to the calculated DOC concentration of 12.8 mM in the test system. Evidently DOC interacts with the surface area of the lipid globules in the emulsion.

In addition, we studied the effects of salts and EDTA on the test system. Inhibition of lipase activity was found for high concentrations of $CaCl_2$ (Table 2) and Na_3PO_4 (10 mM) but no effect was observed for EDTA up to 10 mM at pH 9.0 (data not shown). An increase in lipase activity was measured for NaCl (up to 150 mM) and higher NaCl concentrations were inhibitory to lipase activity at pH 9.0 (Table 2). Obviously, NaCl causes a shift of the apparent pH optimum of lipase activity (Fig. 3). The combination of NaCl and $CaCl_2$ in the assay system resulted in a further apparent inhibition of lipase activity at pH 9.0 (Table 2). The original apparent lipase activity of *ca* 29 nkat mg protein^{-1} was reached with the standard assay containing 150 mM NaCl and 6.6 mM $CaCl_2$ at pH 8.5 (data not shown). With this assay procedure, no lipase activity was detected below pH 7.5.

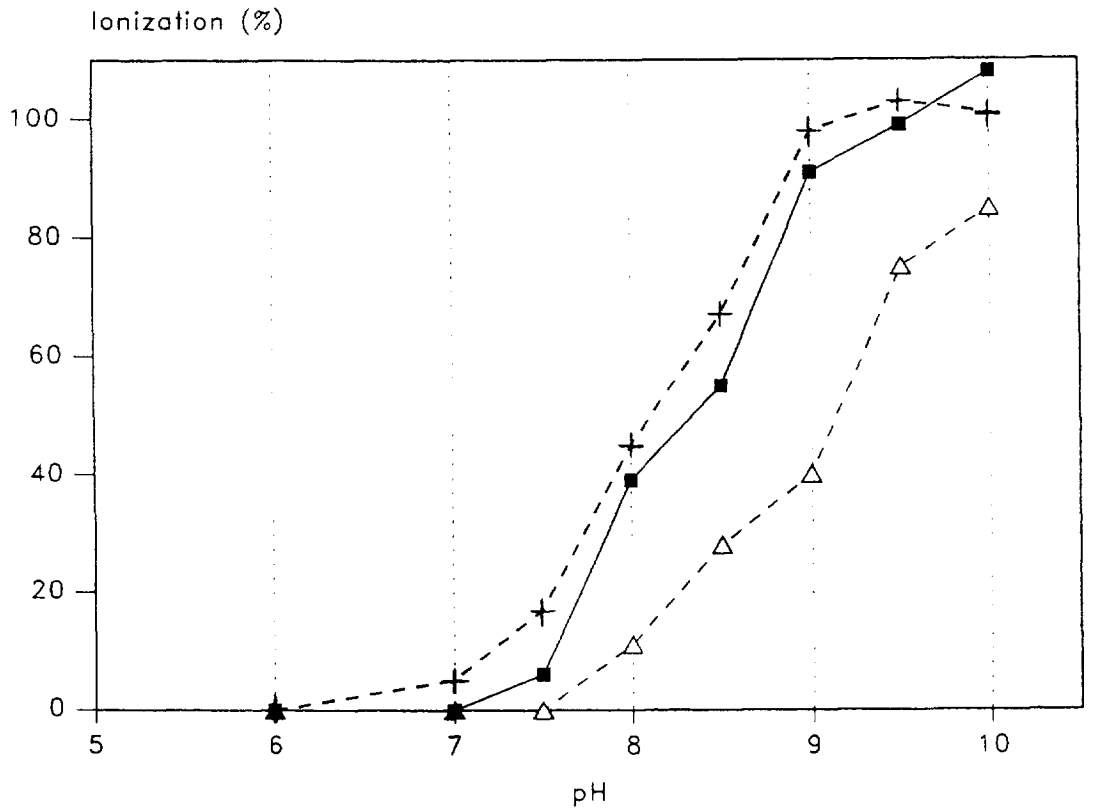

Fig. 2. Titration of oleic acid in aqueous systems and in a stabilized triolein emulsion. (■—■) Oleic acid titrated in triolein emulsion stabilized by both gum arabic and DOC (standard test), (+ – – +) titrated only in the presence of 3% gum arabic and (△ – – △) in the presence of 3% gum arabic containing 2.5 mM DOC.

Table 2. Effects of salts on lipolytic activity of soluble cell fraction. Lipase activity was assayed with stabilized triolein emulsions in the standard system (◀) at pH 9.0

Salts added		Lipase activity (nkat mg protein^{-1})
NaCl	0 mM	20.3
	10.8 mM	29.5 ◀
	37.5 mM	34
	75.0 mM	36
	110.0 mM	39
	150.0 mM	41
	200.0 mM	36.5
	300.0 mM	32.5
$CaCl_2$	0 mM	24.5
	0.5 mM	29
	1.0 mM	31
	2.0 mM	23
	5.0 mM	19
	10.0 mM	7.5
NaCl	150 mM	
plus $CaCl_2$	0.7 mM	45.5
	3.3 mM	39.5
	6.6 mM	20.4

Addition of detergents to the assay medium is likely to affect the substrate-water interface. Lipase from the soluble fraction of rapeseed cotyledons was concentrated by PEG-400 precipitation and resuspended in buffered solutions containing different detergents. Lipase activity in these enzyme preparations was clearly enhanced by the addition of detergents, including DOC (Table 3). For example, up to 0.1% Triton X-100 in the enzyme preparation did not decrease the apparent lipase activity (data not shown).

In summary, at least two effects of DOC on lipase activity were found. 1) An interaction with the interface of the oil-in-water emulsion; and 2) a direct activation of the enzyme.

With the optimized pH-stat method, a total lipase activity of 4.3 nkat pair of cotyledons^{-1} was estimated in crude homogenates (supernatant 1000 *g*) of cotyledons from 4-day-old rape seedlings. This enzyme activity was identical in crude extracts buffered with Tricine–NaOH, pH 7.5, Hepes–NaOH, pH 7.5 or Bicine–NaOH, pH 9.0. Also, non-fatty hypocotyls of 4-day-old rape seedlings were examined for lipase activity. The results given in Table 4 show that lipase activity is also present in a non-fatty tissue. After centrifugation (35 000 *g*), most of the lipase activity was precipitated, reaching a specific activity of 10.5 nkat mg protein^{-1} in the sediment.

DISCUSSION

Lipolysis taking place at an oil–water interface does not obey classical kinetics; enzyme activity evidently depends on interfacial area. Therefore, a sufficiently emulsified long-chain fatty acid ester in the form of triolein allows the use of high substrate concentrations in the continuous monitoring titrimetric assay. Nevertheless, these emulsions are not stable in a thermodynamic sense [1, 11]. Prevention of coalescence of lipid globules in emulsions may be achieved by charging, sterically-stabilizing the surface or by particles adsorbed to the interface [11]. We overcome this technical difficulty by emulsifying triolein with sonic energy in the presence of the stabilizer, gum arabic, and mixing the dispersed substrate with the surface-active agent, desoxycholate. The emulsion is thus stabilized against breakdown both by steric stabilization with gum arabic and, in addition, through charge stabilization by desoxycholate. The use of high concentrations of substrate and emulsifiers resulted in a reproducibly high level of lipase activity. Total lipase activity determined under optimized conditions was between 20-fold and 200-fold higher than reported previously for crude homogenates of cotyledons from rape seedlings [12, 13], measured by a colorimetric method. Lipase activity in crude homogenates was not affected by different buffer systems used for homogenization of the cotyledons.

Common to both the titrimetric method and the copper soap assay method, is that triacylglycerols are emulsified in gum arabic. However, DOC cannot be included in the colorimetric assay, because it yields high background values when quantifying FFA [13]. On the other hand, it was reported that the highest reaction rate for rape seed lipase was reached at 2 mM substrate in the colorimetric assay [12]. Previous determinations and characterizations of lipase activity from rape seedlings were based on an assay with 0.25% gum arabic and 2.5 mM dispersed triolein [14]. The present continuous titrimetric assay contains 33 mM emulsified triolein in 3% gum arabic. As a consequence, a higher surface area of the emulsion is present. Therefore, a more sensitive determination of the FFA released is possible and higher apparent lipase activities are detected. The detection of lipase activity in non-fatty hypocotyls of rape seedlings indicates the improved sensitivity of the assay.

A further advantage of the pH-stat method is the possibility of adding DOC to the assay medium. The DOC solution stabilizes the substrate emulsion, activates the enzyme and may even be important for the binding of lipase to the substrate surface. On the other hand, DOC prevents the accumulation of the released FFA at the water–oil interface of the emulsion. Lipase activity has been reported to be inhibited by the accumulation of FFA in the test system [15]. Our data could also provide a simple explanation for the differences in the characteristics of lipase from rape seeds documented previously [12–16]. They may be due to the different test methods used.

The complex mechanism of lipolysis at the interface between water and a water-insoluble substrate offers many possibilities to explain the effects of salts and other compounds. Direct interaction with the substrate surface or with the FFA liberated, compensation of the negative electrostatic charge, promotion or reduction of the adsorption mechanism of the enzyme onto the surface and enzyme stabilization are possible interpretations. Therefore, results obtained about the characteris-

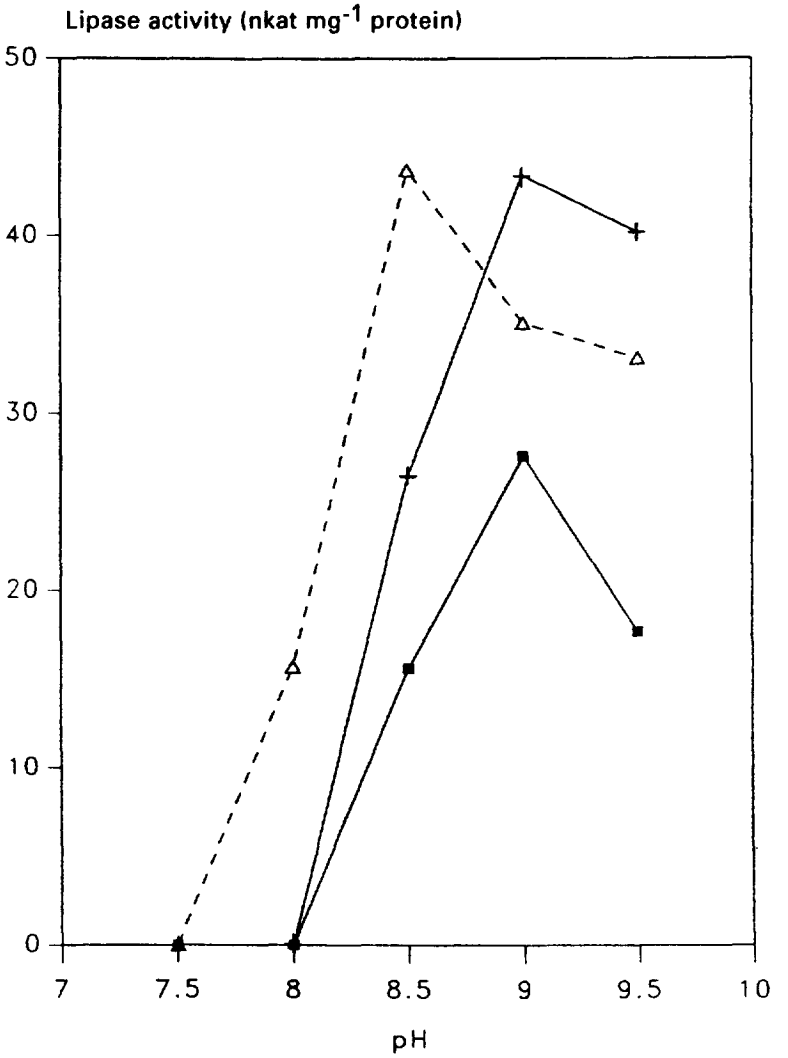

Fig. 3. Effect of NaCl on activity of lipase as a function of pH value. (■—■) Lipase activity measured with the standard assay, (+—+) in the presence of 150 mM NaCl and (△--△) 300 mM NaCl added to standard test.

Table 3. Effects of detergents on a lipase preparation obtained by PEG-400 precipitation from the soluble cell fraction. Precipitated material was resuspended in buffer with or without detergent (100% = 80.5 nkat mg protein^{-1})

Reagent added	Lipase activity (%)
No detergent	100
Octylglycoside (5 mM)	106
Triton X-100 (0.02%)	130
Desoxycholate (1.5 mM)	122

Table 4. Lipase activity in subcellular fractions of hypocotyl extracts from rape seedlings

Fraction	Total lipase activity (nkat)	Specific activity (nkat mg protein^{-1})
Crude homogenate	106	2.7
Pellet (35 000 *g*)	90.5	10.5
Supernatant (35 000 *g*)	0	0

tics of lipase have to be interpreted with care. For example, to remove liberated FFA from the titration system, the pH must be above pH 8.5 in order to achieve complete ionization of the FFA [1, 17]. Thus, the continuous titrimetric methods for the determination of lipase activity operate best at pH 9.0. As a consequence, the pH optimum for rape seed lipase is found to be pH 9.0, as reported [16], although it might reflect only the maximum conditions for showing FFA.

Additional agents in the test mixture may also influence the ionization and release of FFA. Inhibition of lipase activity is often observed, but may be induced by changing parameters for the detection of the FFA released. Ca^{2+} readily reacts with FFA and precipitates FFA at the interface. Also, DOC was precipitated by Ca^{2+} at concentrations higher than 2 mM [18]. As a consequence, the destabilization of the emulsion should be taken into consideration. On the other hand, a direct interaction of Ca^{2+} with the enzyme cannot be excluded. It is difficult to explain the role of Ca^{2+} in our

test system. Sodium ions are well known to suppress inhibition by interfacial charge effects, to promote adsorption of the enzyme and to stabilize the active centre of pancreatic lipase [19]. Lipase activity of rape seedlings was also activated by NaCl (up to 150 mM) in the titrimetric assay but not affected in the colorimetric test system [12]. The decrease in the activity at higher concentrations of NaCl was due to a shift of the pH optimum to pH 8.5. We did not find inhibitory effects of detergents as previously reported [12–14], in fact, the opposite. The effects of salts and detergents on lipase activity observed in this study are in good agreement with previously reported data when lipase was assayed by a trimetric method [15], but are in contrast to reports where lipase activity was determined by the copper soap method [12–14].

In addition, in non-fatty hypocotyls of rape seedlings, lipase activity was detected for the first time, when our optimized assay was employed. So far, lipase activity in non-fatty plant tissues has only been reported for *Elaeis guineensis* [20] and *Zea mays* [21].

EXPERIMENTAL

Homogenization and preparation of cellular fractions. Cotyledons (10–15 g) or hypocotyls (*ca* 30 g) of etiolated 4-day-old rape seedlings (*Brassica napus* L. cv. 'Rubin') were homogenized with a pestle and mortar in 150 mM MES–NaOH, pH 6.5, 10 mM KCl, 1 mm $MgCl_2$, 1 mM EDTA, 2 mM DTT in buffered (0.5 M) sucrose. The homogenate was fractionated by centrifugation at 1000 *g* (10 min) yielding a crude homogenate. This was cleared at 159 000 *g* (90 min) and the supernatant (sol. fr.) was used for most studies, in addition to a protein fr. obtained by PEG-400 precipitation of this supernatant, which was mixed with PEG-400 (15%; v/v), incubated at 4° (60 min) and the insol. material precipitated at 25 000 *g* for 25 min. The sediment was resuspended in a small vol. of 20 mM Tricine–NaOH, pH 7.5 (1 mM EDTA, 1 mM DTT) containing the detergents given in the text and cleared by additional centrifugation (10 000 *g*, 10 min). The crude hypocotyl homogenate was centrifuged at 35 000 *g* (30 min). After centrifugation, the sediment of the hypocotyl extract was resuspended in homogenization buffer and, in addition to the supernatant, used for the tests described.

Assay for lipase activity. Lipolytically-released FFA were determined by a pH-stat technique as described previously [9, 16, 22]. Triolein (pure, 100 mmol, 2 ml) was emulsified in gum arabic (10% w/v, 16.5 ml) by sonication (45 sec, 250 W, 4°). Emulsions were stirred, adjusted to pH 8.5–9.0 and then a DOC soln (1.6% w/v DOC; 32 mM NaCl) was added in drops to the emulsion and the pH value adjusted by addition of NaOH. The emulsion (4 ml) and 2 ml H_2O were adjusted to 37° in a reaction vessel finally containing 33 mM emulsified triolein, 12.8 mM DOC and 10.7 mM NaCl. The FFA released by the action of lipase were titrated at pH 9.0 by 0.01 N NaOH and monitored for up to 15 min. Salts were added in different amounts to this standard assay. In all cases, continuous acidification of the text mixture depended on both the triolein content and the amount of protein. Lipase activities are corrected against nonenzymatic acidification reactions in the test system. The protein content of cellular frs was calculated by the method of ref. [23].

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