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Full Length Research Paper

Immunopurification of a rape *(Brassica napus* L.) seedling lipase

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Lipase or triacylglycerol acylhydrolase (E.C.3.1.1.3) was purified to homogeneity from rapeseed-germinated cotyledons (*Brassica napus* L.). The purification scheme involved homogenization, centrifugation, ultracentrifugation and affinity chromatography using polyclonal antibodies raised against porcine pancreatic lipase. The purified rapeseed lipase was homogenous and did not contain contaminating proteins detectable by SDS-PAGE and HPLC analysis. The specific activity of the purified preparation was increased about 1950 times, with an overall yield of 35%. The rapeseed lipase was found to be a cytosoluble, glycosylated and heat-labile serine-hydrolase. It was monomeric with a molecular mass of 38 kDa and a pI of 6.6. The purification method used in the present work is rapid, simple and yields highly purified lipase. It may therefore be applicable in the purification of other uncharacterized plant lipases.

Key words: *Brassica napus* L., immuno-affinity, lipase, purification, triacylglycerol acyl hydrolase.

INTRODUCTION

Interest in lipases (triacyl glycerol acylhydrolases: E.C.3.1.1.3) from micro-organisms, animals and plant sources has markedly increased in the last decade owing to their novel and multifold applications in industry, oleochemistry, organic synthesis, detergent formulation, nutrition and medicine. Lipases are unique in catalyzing the hydrolysis of fats into fatty acids and glycerols at the water-lipid interface and reversing the reaction in non-aqueous media. The comparison of the properties of these proteins provided valuable information on their evolutionary relationships. In addition, their structure and their function analysis may help us to understand the fundamental mechanisms in this family (Kanaya et al., 1998). Recently, the alignment of animal, bacterial and fungal lipase sequences suggested the presence of sequence homologies including a significant conserved region, Gly-X-Ser-X-Gly as the catalytic moiety (Kanaya et al., 1998; Saxena et al., 2003; Osterlund et al., 1996). This consensus sequence, found in the substrate binding site, contains a serine residue suspected to be essential for binding to lipid substrates.

Lipases from a large number of bacterial (Gotz, 1991; Tyski et al., 1983; Vicente et al., 1990; Aires-Barros and Cabral, 1991), yeast (Veeraragavan and Gibbs, 1989), fungal (Suzuki et al., 1986; Kundu et al., 1987; Sugihara et al., 1988; Isobe and Nokihara, 1991) and animal sources (Kanaya et al., 1998; Osterlund et al., 1996; Cambillau and Bourne, 1991; Cheng et al., 1985; Carriere et al., 1994) have been purified to homogeneity, but not from plants at our knowledge. Compared to bacterial, fungal and animal lipases, little is known about plant lipases. This lack of information about plant lipases could be due to their complicated and laborious purify-cation (Weselake and Jain, 1992). The difficulties involved in carrying out research on plant lipases, particu-larly from oil seeds, have been essentially attributed to their low abundance, their instability and the loss of activity using the traditional purification strategies. These procedures were generally based on clarification, preci-pitation, differential and density gradient centrifugations followed by sequential combinations of different chroma-tographic steps resulting in a low final yield (Ben-Hamida and Mazliak, 1985). Many different methods have been

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Abbreviations: BHT; Butylhydroxytoluene **DTT;** Dithiotriethol, **EC-lipase;** Euphorbia characias lipase, **IRS-lipase;** Immunopurified rapeseed lipase, **HPL;** Human pancreatic lipase, **PPL;** Porcine Pancreatic Lipase, **TAGs;** Triacylglyceols.

applied to purify plant lipases without reaching homogeneity (Huang, 1993).

To overcome these difficulties, we have tried to apply a new technique essentially based on an immunopurification step to prepare the rapeseed lipase. Antibodies immobilized onto support matrices have been already used to immunopurify a wide variety of biological compounds such as bacterial proteins (Sjoberg and Holmgren, 1973), enzymes (Melchers and Messer, 1970), hormones (Murphy et al., 1973), viral proteins (Diaco et al., 1986) and virus particles (Kenyon et al., 1973).

We report here the immunopurification and characterization of a rapeseed lipase by affinity chromatography using an immunosort prepared by covalently coupling polyclonal antibodies raised against the commercial porcine pancreatic lipase (anti-PPL) to activated CNBr-Sepharose 4B. In a previous work, we found that these anti-PPL antibodies cross-react with the rapeseed lipase (Belguith et al., 2001).

MATERIALS AND METHODS

Plant material

Rapeseed (*Brassica napus* L.), cultivated locally, were soaked for 24 h, then, surface sterilized with 5% (v/v) $CaCl_2O_2$ in distilled water for 5 min. Sterilized seeds were allowed to germinate on filter paper moistened with distilled water in darkness at 26°C.

Homogenization and fractionation

The rape (*Brassica napus* L.) seedling lipase was prepared as described in a previous work [26]. Briefly, cotyledons (20 to 30 g) from 3-days old seedlings were excised from the hypocotyls and homogenized using a pestle and a mortar in grinding buffer containing 0.15 M Tris, pH 7.5, 0.6 M sucrose, 1 mM DTT, 1 mM benzamidine and 10 mM KCl at a ratio of 1:5 (w/v). The resulting homogenate was filtered through a Miracloth layer and fractionated by centrifugation at 10,000 g for 30 min into a yellow fatty layer (removed carefully with a spatula), a supernatant solution (S10) and a pellet. The S10 fraction was centrifuged at 100,000 g for 3 h. The resulting supernatant (S100) was stored at -20°C. All the homogenization and centrifugation steps were performed at 4°C.

Lipase activity assay

Colorimetric method

Lipase activity was determined by measuring free fatty acids produced by triacylglycerol (TAGs) hydrolysis using a colorimetric method (Duncomb, 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, 1 M Tris- HCl 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.

Radioactive method

In radiometric assays, lipase activity was measured using TAGs containing radiolabelled acyl chains (243 cpm/µl) as a substrate (Beisson et al., 1999). 20 µl of the rapeseed lipase preparation was incubated at pH 7.5 in the presence of 10 µl of radiolabeled triolein (22 Ci/mM) (Perkin-Elmer), 1% sodium taurodeoxycholate (NaTDC) and 7.6 µl of 4 M $CaCl_2$, in a final volume of 200 µl. After each 15 min, 50 µl of the reaction mixture were added to 1 ml of the stopping buffer. Then, a mixture of methanol/chloroform/heptane (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 resulting of the hydrolysis of radiolabeled TAGs was counted on a Beckman LS 1801 apparatus.

Fluorimetric method

Fluorescent TAGs were extracted from the seed kernels of *Parinari glaberrimum*. 5 mg 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) and stored in the dark at –20 ^{0}C under an argon atmosphere.

After evaporating ethanol under a nitrogen stream, 3 mg of purified TAGs was placed in a 0.5-ml polypropylene microtube, 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 $CaCl_2$, 0.001% (w/v) 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).

The incubation buffer (990 µl) and the TAGs stock solution (10 µl) were added consecutively to a quartz cuvette of 1.5 ml (optic path-length 1 cm) containing a magnetic stirring bar 8 mm. The final TAGs concentration was 18 µg/ml. The above mixture is 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 due to the free fatty acids released 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 (Beisson et al., 1999).

The following standard incubation buffer (pH 8) was used: 4 mM NaTDC, 100 mM NaCl, 6 mM $CaC1_2$, 0.001% (w/v) BHT.

Electrophoresis

SDS-PAGE (Laemmli, 1970) was carried out on a discontinuous 6% stacking and 12% resolving polyacrylamide gel with Tris-Glycine running buffer and staining with Coomassie Brilliant Blue R-250 or silver nitrate. Standards (Sigma, St. Louis, MO, USA): lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), albumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase (97 kDa). The native-PAGE was done in the same gel and buffer conditions without SDS. Total proteins were quantified assayed according to the Bradford method (Bradford, 1976) using bovine serum albumin (Sigma) as standard.

HPLC analysis

Reversed-phase HPLC (C8 column Ultra sphere octyl 5 μm; 25 x 0.46 cm), using a linear gradient of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid, was used to analyze the active immunopurified fraction.

Polyclonal antibody production

500 µl (0.5 mg protein) of porcine pancreatic lipase type VI-S (Sigma, St. Louis, MO) mixed with an equal volume of complete Freund adjuvant was injected into rabbits. 2 further injections were given fortnight intervals with the same amount of the immunogen emulsified in incomplete Freund adjuvant. 2 weeks after the booster injection, sera samples were collected in order to evaluate the immune response of the rabbits, using ring test and ELISA as described later. Another injection was carried out after 5 weeks and the animals were bled 2 weeks after that last injection.

Direct-binding ELISA test

Enzyme-linked Immunosorbent assay was performed using the method of Kang et al. (1988). For all ELISA procedures the following buffers were used. Coating buffer: PBS. Wash medium: PBS-Tween-20 (0.5 g/L). Saturating buffer: PBS-Tween-20 containing bovine serum albumin (BSA; 5%). Substrate solution: O-phenyllene-diamine (0.4 g/L) (Sigma, St. Louis, MO) dissolved in 0.05 M sodium phosphate/citrate buffer, pH 5, containing hydrogen peroxide. Stop solution: 3 M HCl.

Immunoprecipitation

To immunoprecipitate lipases (IRS-lipase and HPL), 20 µl of lipase was added to 20 µl of anti- EC-A lipase immune serum at a 1:500 dilution. The total mixture were incubated at 37°C for 1 h, immunoprecipitate was centrifuged at 10,000 x g for 10 min and residual lipase activity remaining in the supernatant was determined by the fluorimetric and radiolabeled method.

Immunoaffinity chromatography column

A CNBr-Sepharose 4B (Pharmacia Biotech AB) pre-activated gel was used to prepare the immunoaffinity column. 20 mg of the purified IgG anti-PPL was dissolved in the coupling buffer 0.1 M Na HCO3 pH 8.3 containing 0.5 M NaCl.

The anti-PPL antibodies were first coupled to the CNBr-Sepharose 4B pre-activated gel according to the manufacturer's instructions and all free antibodies were removed by washing and BSA (3%) was used to block unreacted gel groups. To control the efficiency of the adsorbance of antibodies to the gel, we measured the absorbance at 280 nm of the eluted fraction. Proteins of the S100 fraction (10 ml) concentrated by acetone precipitation were taken up in binding buffer (0.1 M $NaHCO_3$, pH 8.2), applied to the immunoaffinity column equilibrated with the same buffer and allowed to react overnight at 4°C with the immunoadsorbent to optimize the fixation of the rapeseed lipase by the antibodies Anti-PPL. The column was washed with binding buffer (4 x 5 mL), to remove unbound proteins.

To elute bound proteins, the column was washed with glycine buffer 0.25 M pH 2.2 and in order to make the pH close to the neutrality, each eluted volume was supplemented with 50 µl of Tris-Hcl 1 M (pH 8). The eluted fractions were submitted to a lipase activity test and a protein quantification test (measurement of the absorbance at 280 nm).

RESULTS

Enzyme purification

Rapeseed lipase activity was found in the soluble fraction of 3-day old seedlings. After ultracentrifugation at 100,000 x g approximately 70% of rapeseed lipase activity was remained in the supernatant S100 (Belguith et al., 1999). In the present work we used an immunoaffinity chromatography to purify rapeseed lipase from the soluble fraction S100.

The S100 proteins concentrate was subjected to immunoaffinity chromatography on immobilized polyclonal antibodies against PPL, which had been demonstrated in a previous work to cross react with the rapeseed lipase (Belguith et al., 2001).

Figure 1 shows the elution profile of rapeseed lipase with 0.2 M glycin buffer, pH 2.2, from the antibodies Sepharose column. Protein quantification and lipase activity test were performed for each fraction, only in the fractions 4, 5, 6 and 7 a lipase activity was detected.

The overall purification protocol is summarized in the Table 1. The most striking aspect of the procedure is that the immunopurification step yielded relatively little protein, but with a specific activity of 91.83 nkat.mg-1. The enzyme preparation eluted from the immunoaffinity column which corresponded to a yield of about 35% and a 1953-fold enrichment of lipase activity of the original fraction (Table 1), was homogeneous as proved by SDS-PAGE analysis. The polypeptide eluted from the immunoaffinity column migrated as a single peptide protein species upon SDS-PAGE at approximately 39 kDa (Figure 2). This result suggests that the IRS-lipase is a monomeric protein with a molecular weigh about (39 kDa) similar to the Euphorbia characias lipase (Moulin et al., 1994).

In order to evaluate the purity of the immunopuri-fied rapeseed lipase (IRS-lipase), the active immunopurifiedfraction was analyzed by reverse phase HPLC. A single and symmetric peak was eluted at about 50% acetonitrile and eluted at 19.72 min (Figure 3). The HPLC profile confirms that IRS-lipase was homogeneous. Our results demonstrate the efficiency of the immunoaffinity chromatography to purify plant lipases. The immunoaffinity column allowed us to purify the immunoaffinity column allowed immunoaffinity column allowed us to purify the rapeseed lipase with a high purification factor.

This type of strategy may be useful in the purification of low abundance plant proteins such as lipases.

Biochemical characteristics of cytosolic rapeseed lipase

In a first step, different tests were used to evaluate and to confirm that the IRS-lipase is a true triacylglycerol acylhydrolase (E.C.3.1.1.3) of germination. In a second step we have characterized the IRS-lipase. All experiments were carried out in triplicate.

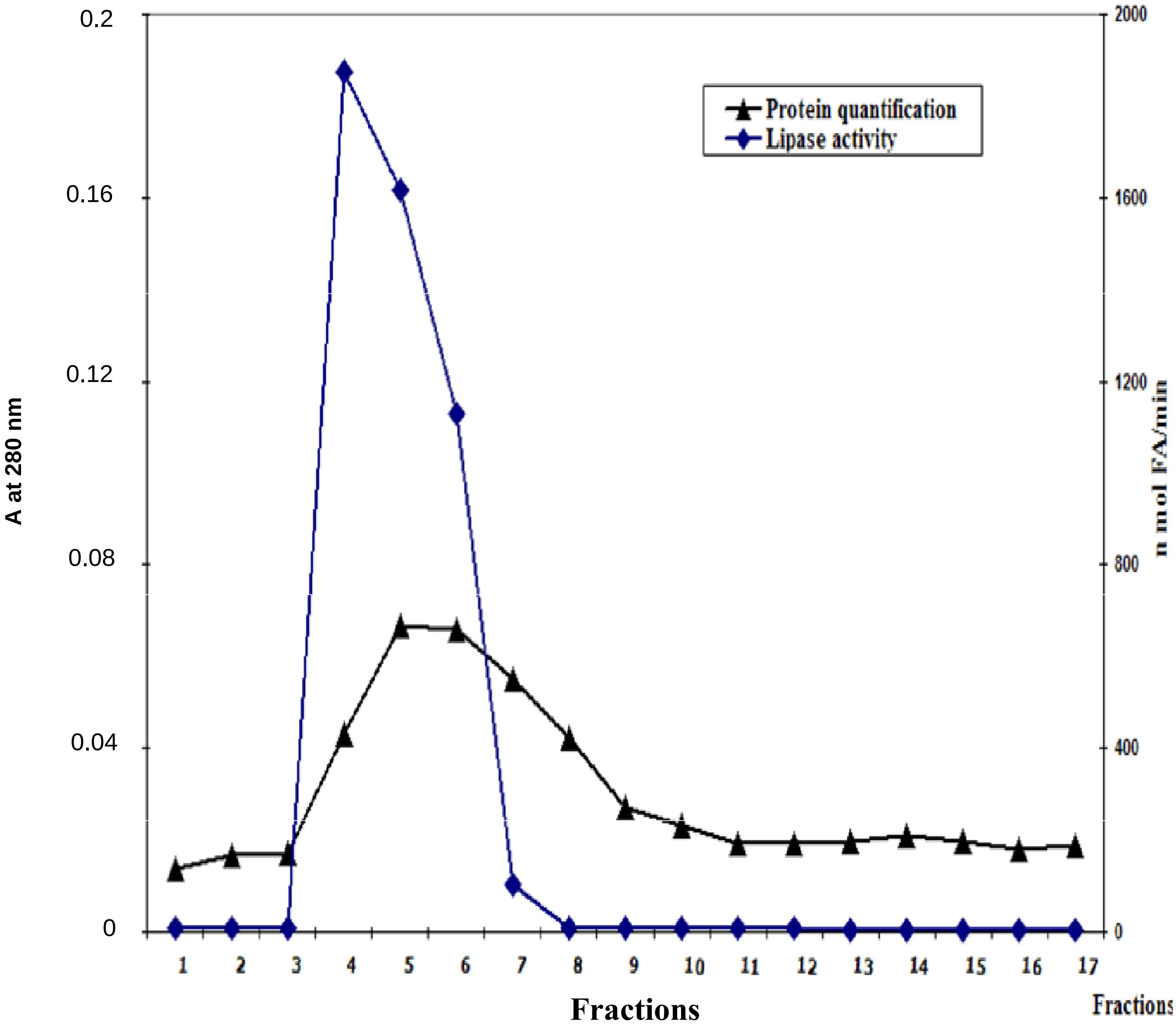

Figure 1. Elution profile from affinity chromatography on CNBr- activated sepharose-4B column. Enzyme preparation (S100) was applied onto the column with stepwise elution with 0.2 M glycin buffer, pH 2.2. Protein quantification and lipase activity test were performed for each fraction.

Table 1. Effect of treatments on separation of seeds from wild pomegranate and their chemical characteristics.

Treatments	Time taken for separation (min)	Labour saving (%)		Titratable reducing ascorbic acid			
		Excluding treatment time	Including treatment time	pH	Acidity (%)	Sugar (%)	(mg/100 g)
CT	13.6 (±5.4)	–	–	6.63 (±0.39)	2.8 (±0.20)	7.2 (±0.2)	19.20 (±0.65)
SR1	8.3 (±3.7)	39.0	3.40	6.16 (±0.16)	3.0 (±0.20)	7.8 (±0.4)	17.70 (±0.71)
SR2	5.8 (±2.6)	57.0	6.02	6.13 (±0.11)	3.1 (±0.23)	7.9 (±0.4)	17.20 (±0.38)
HW1	3.4 (±2.0)	75.0	60.0	6.03 (±0.10)	3.1 (±0.21)	7.8 (±0.2)	19.60 (±0.60)
HW2	2.5 (±1.7)	80.0	74.0	6.10 (±0.13)	3.0 (±0.10)	7.8 (±0.1)	19.47 (±0.55)
WS1	10.0 (±3.0)	–	–	6.70 (±0.16)	2.9 (±0.15)	7.2 (±0.3)	19.30 (±0.75)
WS2	12.1 (±4.1)	–	–	6.80 (±0.20)	2.8 (±0.20)	7. 1 (±0.1)	19.56 (±0.45)

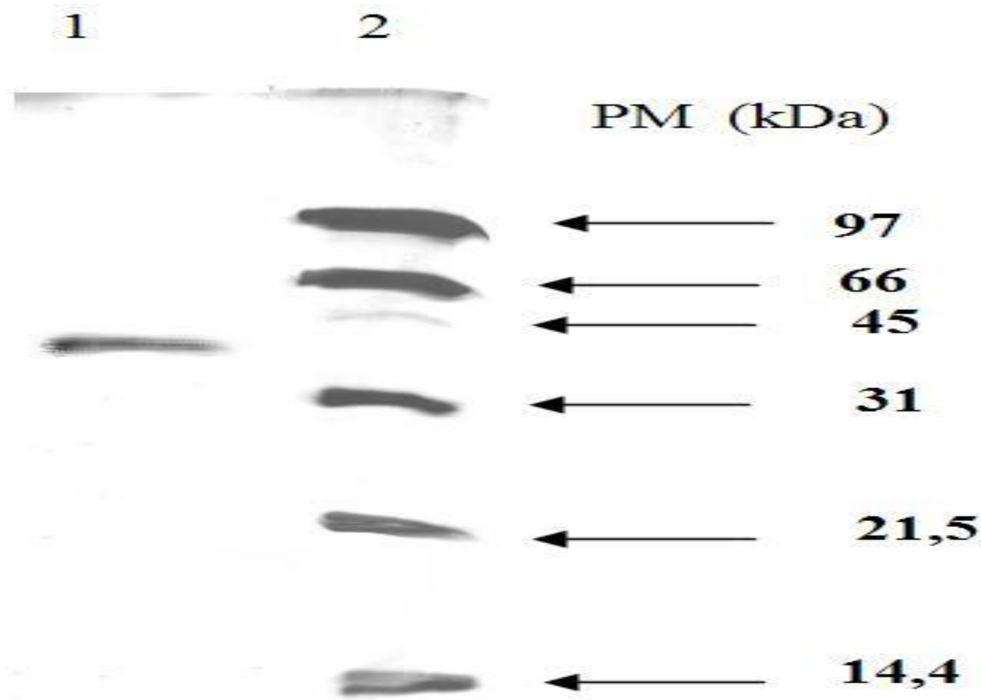

Figure 2. SDS-PAGE analysis of the immunopurified rapeseed lipase on a 12% gel, stained with silver nitrate. Lane 1: 12 μg of IRS-lipase, lane 2: molecular mass markers.

Lipase activity tests

In order to confirm that the IRS-lipase is a true triacylglycerol acylhydrolase (E.C.3.1.1.3) of germination, we tested the lipase activity using 2 different methods.

Results obtained using radiolabeled TAGs

Lipase activity was measured using TAGs containing radiolabelled acyl chains (243 cpm/μl) as a substrate. We quantify the cpm resulting of the hydrolysis of radiolabeled TAGs. Figure 4a clearly shows that the IRS-lipase presents a high activity level with a specific activity of about 94 nkat.mg^{-1}.

We observe a linear kinetic for 15 min, after that it reaches a plateau. No activity was observed in the heat-treated IRS-lipase for 5 min at 90°C used as a control, these results also suggest that the IRS-lipase is a heat-labile enzyme.

Result obtained using fluorimetric method

Lipase activity was measured using a *Parinari glaberrium* TAGs as a substrate, the fluorescence was recorded every 10 min for 3 h. Figure 4b shows that the increase of relative fluorescence was linear with time during 120 min of incubation, with a specific activity of about 92 nkat.mg^{-1}, after that it reaches a plateau. These results are identical of the obtained ones using the radiolabeled method.

Esterase activity test

This test was performed to investigate if the IRS-lipase presents or not an esterase activity such as some described partially purified plant lipases. Figure 5 shows the result of the separation by native-PAGE of the IRS-lipase and the crude 3-days germinating cotyledons extract, submitted after that to a specific revelation using the α-naphtol acetate as a substrate. The obtained electrophoregramme revealed three esterase isoforms in the crude extract (lane 2 and 3) as found in the sunflower germinating crude extract (Ben Hamida, 1986) and in the excised sunflower cotyledons crude extract. No esterase activity was detected in the IRS-lipase fraction (lane1). This result indicates that the IRS-lipase did not exhibit any esterase activity.

The different methods used to test lipase activity confirm that the immunopurified rapeseed lipase is a true lipase of germination and not an esterase.

Some structure and molecular properties of IRS-lipase

Effect of antibodies anti-*Euphorbia characias* lipase on rapeseed lipase activity

In order to study the effect of the polyclonal anti-*Euphorbia characias* (anti-EC-lipase) antibodies (made by the laboratory of LLE-CNRS Marseille-France) on the IRS- lipase activity, we measured the residual lipase acti

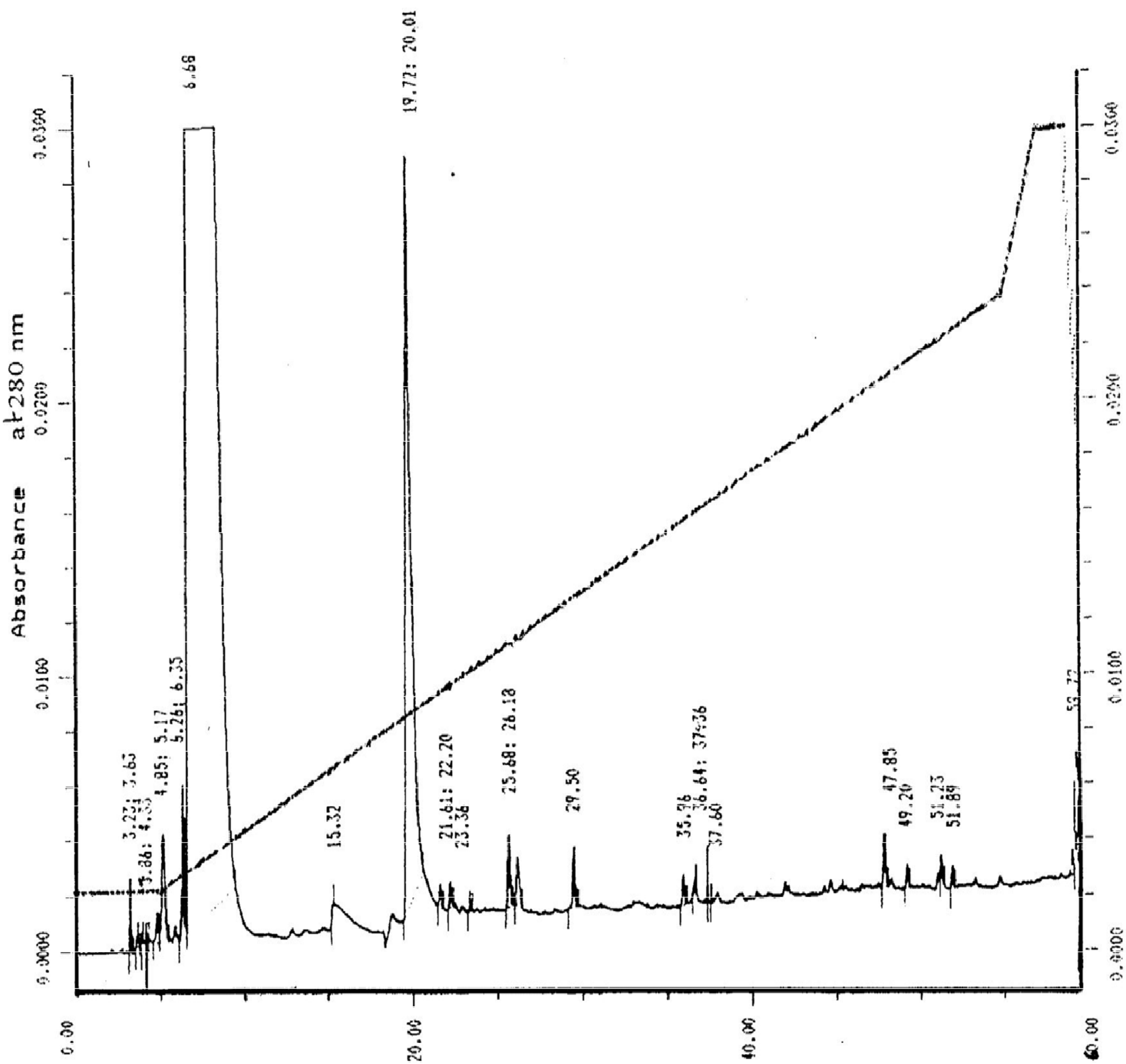

Figure 3. Reversed-phase HPLC analysis of the immunopurified rapeseed lipase. The separation was carried out on C8 column Ultra sphere octyl 5 mm (25 x 0.46 cm), using a linear gradient of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid.

vity using 2 different methods (radiolabeled and fluorimetric) as described in the material and method section. 2 control tests were carried out using pre-incubated IRS-lipase with a pre-immune rabbit serum and a heat-treated IRS-lipase for 5 min at 90°C. In these cases, no change in the lipase activity amount was observed, but a very low residual lipase activity was found in the recuperated supernatant (S10) with a 97% inhibition rate (Figure 6). These results show evident immunochemical cross-reactivity between EC-lipase and rapeseed lipase, suggesting that these antibodies recognized the native IRS-lipase and bound to some residues located in the catalytic site

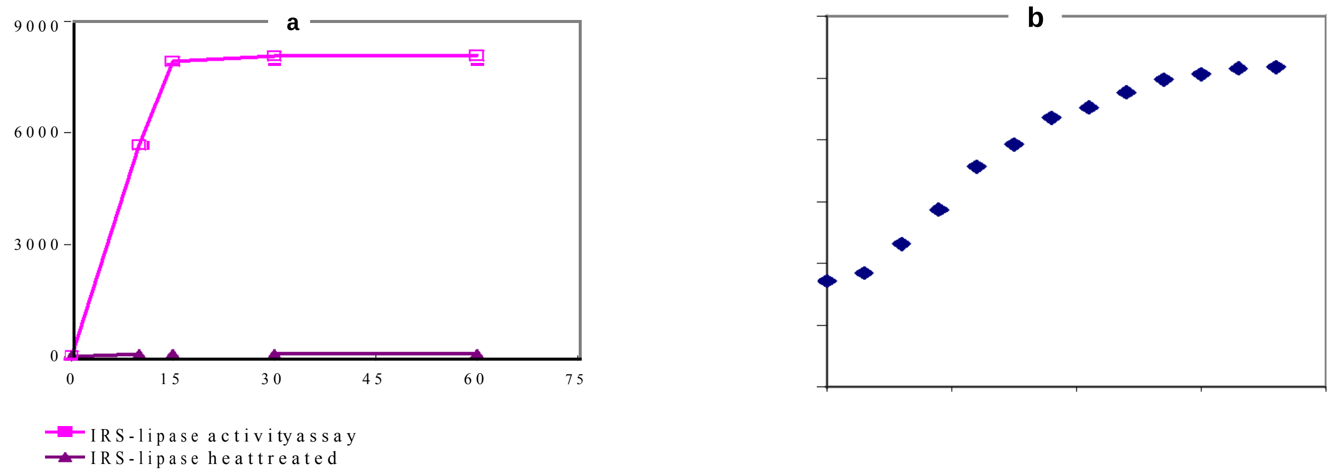

Figure 4. Test of the IRS-lipase activity using 2 methods. (a): Radiolabeled assay, using 20 µl of native and heat treated IRS-lipase were incubated at pH 7.5 and 37°C, in presence of 10 µl of radiolabeled triolein. (b): Kinetics of hydrolysis of naturally fluorescent TAGs from *P. glaberrimum* upon incubation with 20 µl of IRS-lipase incubated 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.

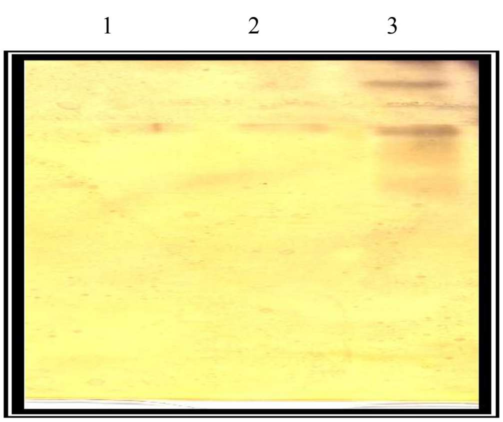

Figure 5. Electrophoregramme of esterases specific revelation. 6 to 12 µg of proteins were separated by native PAGE, the gel was submitted after that to an esterase specific revelation, using α-naphtol acetate as a substrate. Lane 1: 12 µg of ISR-lipase, lane 2 and 3: 6 and 12 µg of the rapeseed crude extract (S10).

or are related to it.

Evidence of a cross-reactivity with *Euphorbia characias* lipase

To confirm the last results, we assessed the immuno-blotting technique, using the anti-EC-lipase polyclonal immune serum at a 1: 500 dilution (Belguith et al., 2001). The immunoblot presented in Figure 7 demon-strates clearly that the anti-ECL antibodies cross-react with IRS-lipase. This result suggests an antigenic relationship between the 2 lipases.

Effect of the tetrahydrolipstain on rapeseed lipase activity

The tetrahydrolipstain (THL) is considered as the first irreversible and selective inhibitor of lipases (Hadvary et al., 1991). 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. 2 cuvette assays were prepared, in the first we incubated the IRS-lipase alone as a positive control and in the second we added 5 µl of THL before 20 min of incubation. We observed (Figure 8) that the increase of the fluorescence intensity measured at 420 nm can be stopped readily by the addition of THL and it reaches a plateau at about 70 (arbitrary units). This inhibition by THL suggests the presence of a serine-residue in the catalytic site of IRS-lipase and that it is a serine triacylglycerol acyl hydrolase as the major of lipases.

Mass spectrometry analysis

Mass spectrometry is considered as an indispensable technique to analyze and characterize the primary structure of proteins. This technique allows us to evaluate the purity and to get the exact molecular weight of proteins. The MALDI-TOF mass specter of the rapeseed lipase shows a single peak with a MW of 38,0387 (Figure 9), this MW is very close to that of EC-lipase described by Moulin et al. (1994).

A short protein sequence of the IRS-lipase was obtained by Edman degradation and the sequence generated was

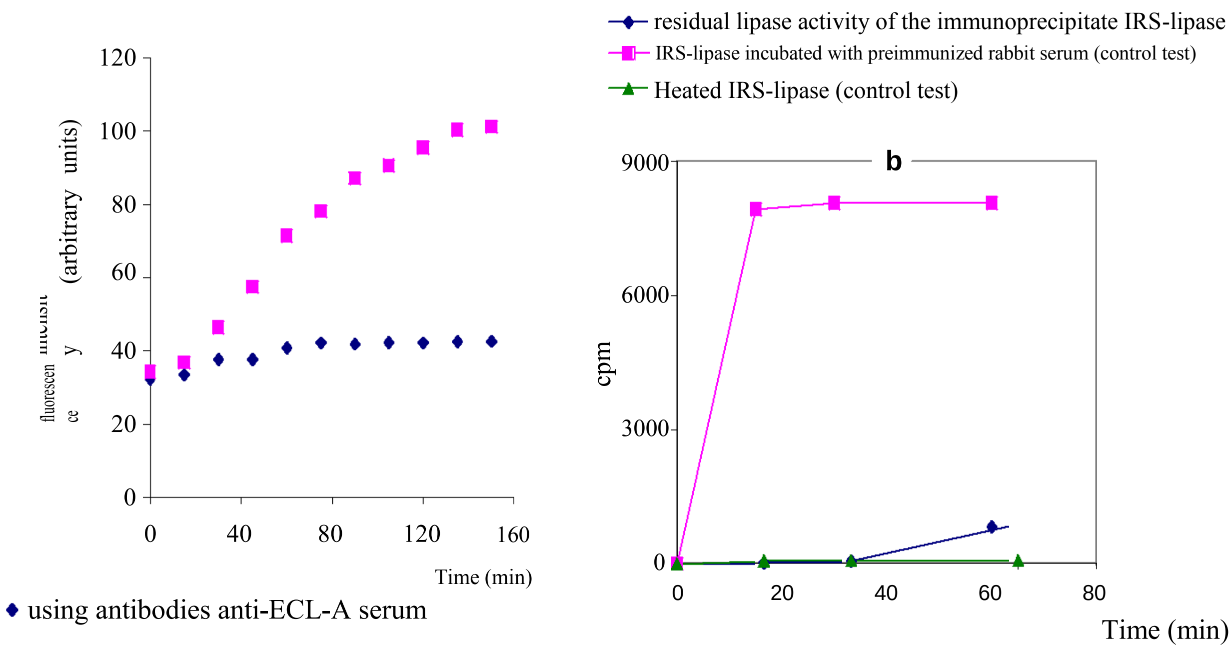

Figure 6. Immunoprecipitation of IRS- lipase and HPL by antibodies anti-EC-A lipase. Residual lipase activity was measured using Fluorimetric method (a) and the radiolabeled method (b).

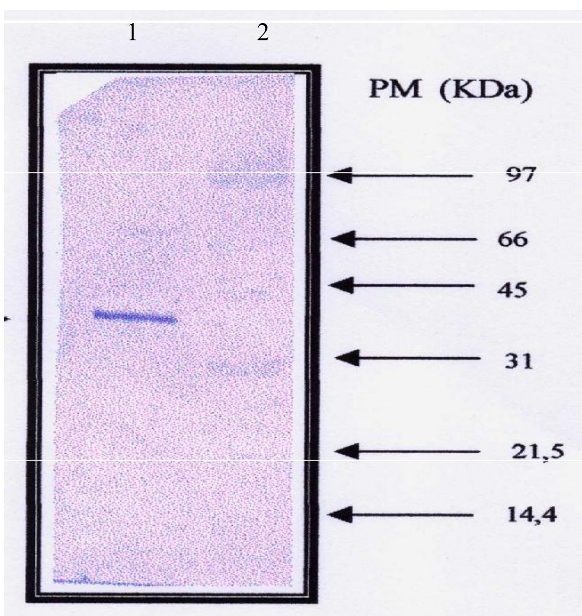

Figure 7. Immunoblot analysis of the IRS-lipase separated by SDS-PAGE, showing a cross reactivity with *E. characias* lipase. The western blot was probed with an anti-EC- lipase polyclonal immune serum at a 1:500 dilution. Lane 1, 15 µg of the IRS-lipase; 2, Protein markers.

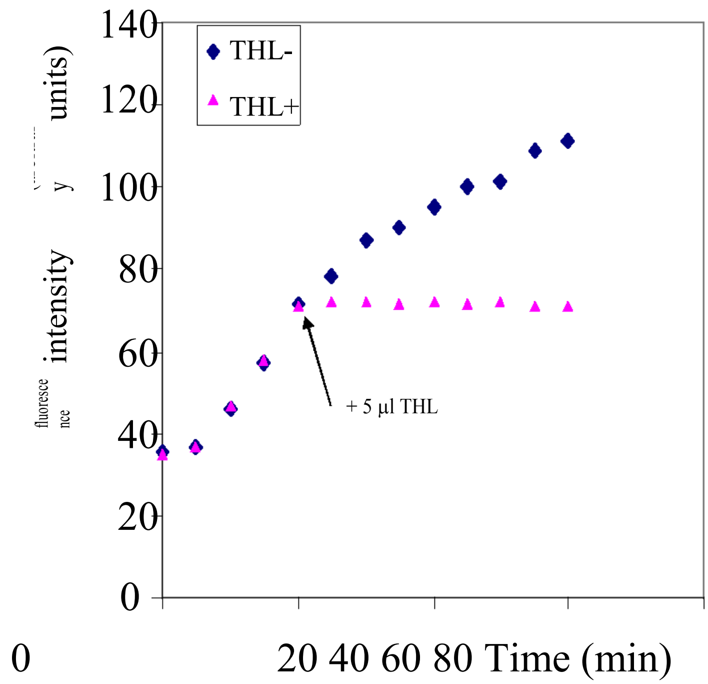

Figure 8. Effect of the addition of tertahydrolipstain (THL) on the IRS-lipase activity. 5 µ l of THL was added in the cuvette assay. Lipase activity was measured using the fluorimetric method.

(D, S)INGGXATLPQX.

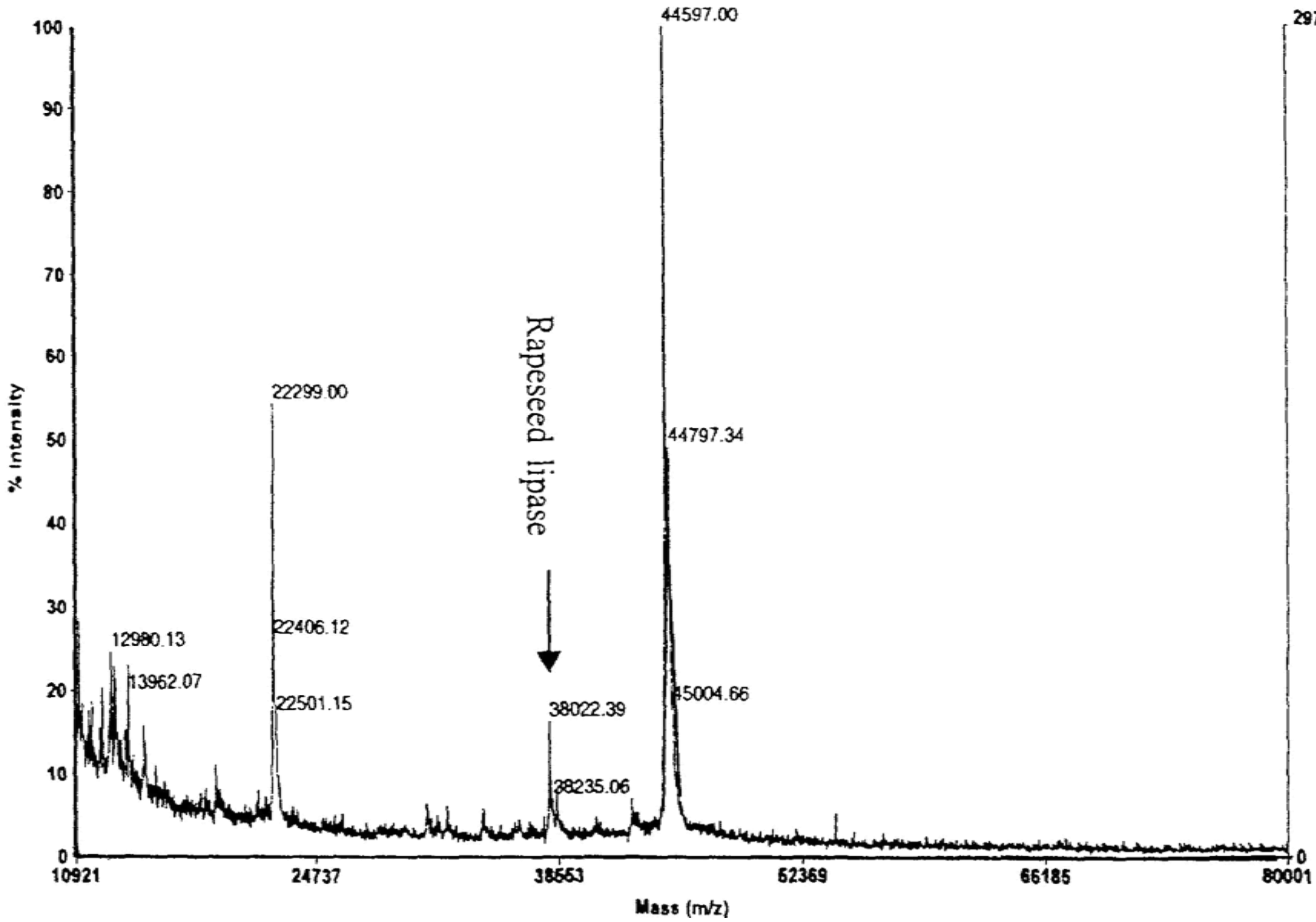

Figure 9. The MALDI-TOF spectrum of the immunopurified rapeseed lipase.

DISCUSSION

We have performed a rapid, high yielding, inexpensive and reproducible procedure for rapeseed lipase purification, it seems to be efficient to purify low abundant plant lipases since the overall obtained yield in our case is about 35.19% with a 1950- fold purification factor. The purity of the immunopurified lipase was firstly tested by SDS-PAGE, a single band was stained in the polyacrylamide gel with a MW of about 39 kDa, secondly by HPLC where a single and symmetric peak was obtained. Finally, using mass spectrometry analysis, we determined that the rapeseed lipase is a monomeric protein having an exact MW of 38.0387 kDa, molecular weight identical to that of EC- lipase. Our results confirm that the immunopurification is one of the most selective and powerful affinity techniques for protein purification leading to purification of 1000 to 10,000- fold in a single step (Harlow and Lane, 1988). Very few examples of immunopurification of lipase are found in the literature.

This method is recommended to be used as a first step on purification procedures leading to high recovery yields. It can be considered as a highly selective purification technique, that require less number of steps leading to an efficient separation and high recovery purification. Immunopurification techniques can be used in industries, because they are rapid, efficient, inexpensive, powerful affinity techniques for protein purification and amenable to large-scale operations.

The different methods used to test lipase activity confirm that the immunopurified rapeseed lipase is a true lipase of germination and not an esterase.

Generally plant proteins are glycosylated and characterized by the presence of a glycane structure related to the asparagines residues. A positive reaction was obtained after incubation of the transferred IRS-lipase nitrocellulose membrane with the biotine conjugated hydrazide and with the alkaline phosphatase conjugated avidine. This result suggests that the IRS-lipase is a glycoprotein (data not shown).

We also demonstrate that the rapeseed lipase is glycosylated and it was inhibited by THL. It is worth noting that the total inactivation of the rapeseed lipase by THL supports the existence of an activated serine in the catalytic site and that is a true serine triacyl glycerol acylhydrolase.

This result suggests a structure similarities and a chemical relationship between lipases from different organisms. This suggestion is consistent with the presence in the primary rapeseed lipase amino acid sequence of the consensus motif Gly-X-Ser-X-Gly. Nearly, all lipases that undergo interfacial activation are somehow akin to a molecular structure covering the catalytic site. Using anti-EC lipase antibodies, we demonstrate an evident immunochemical cross reactivity between EC-lipase and IRS-lipase, reflecting a considerable degree of structure homology between these enzymes. Furthermore, these antibodies were able to total inhibit rapeseed lipase activity suggesting that they bind to some residue located in the catalytic site.

Moulin et al. (1994) had demonstrated that the EC-lipase presents the consensus sequence Gly-X-Ser-X-Gly found in the B chain of the ricin lipase. It seems that the rapeseed lipase as all the purified lipases from different organisms present the consensus sequence as described by Moulin et al. (1994) and that these plant lipases are antigenically very similar.

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