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Full Length Research Paper

Cross reaction between P-61 sunflower seedlings oleosomal protein band and porcine pancreatic lipase

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A true triacylglycerol lipase was detected in germinating sunflower (*Helianthus annuus* L.) seedlings associated to oleosomes. This enzyme that has not yet been identified was partially purified as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) (10%); two protein bands, P-61 and P-66 of 61 and 66 kDa, were isolated from the active lipase fraction. Polyclonal antibodies (PAbs) directed against each protein band were produced. Only PAbs against sunflower P-61 were able to inhibit the total lipolytic activity of our preparation. Hence, only the P-61 protein carries the sunflower lipase activity. The relationship between a pure porcine pancreatic lipase and the P-61 purified sunflower lipolytic fraction was investigated, leading to the first evidence of cross reaction between the porcine pancreatic lipase and the P-61 sunflower lipase fraction. These results are in agreement with common epitopes to the porcine pancreatic lipase and the sunflower P-61.

Key words: Triacylglycerol lipase, oleosomes, polyclonal antibodies, cross-reactivity, *Helianthus annuus* L.

INTRODUCTION

In oilseeds, triacylglycerols (TAG) are stocked in subcellular droplets called lipid bodies or oleosomes surrounded by a monolayer of phospholipids and proteins (Yatsu and Jacks, 1972; Huang, 1996). During the early germination steps, this lipid reserve is rapidly mobilized: lipases (EC. 3. 1. 1. 3.) catalyze triacylglycerol degradation into free fatty acids and glycerol. Plant lipase activities are usually membrane-associated and can be found in oil bodies, microsomes or glyoxysomes (Lin and Huang, 1984; Maeshima and Beevers, 1985; Hammer and Murphy, 1994). The oleosomal lipase from corn scutella (*Zea mays*) and the lipase associated to castor bean "glyoxysomes" (*Ricinus communis*) have been characterized and have respectively a molecular weight of 65 and 62 kDa (Lin and Huang, 1984; Maeshima and Beevers, 1985). However, and despite several studies, our knowledge about these plant enzymes is still insufficient, probably because of their low detected activity *in vitro*.

The gene encoding the well known acid lipase from castor beans (Ory et al., 1962, 1964,196,1968,1969) has been cloned and sequenced (Eastmond, 2004). The molecular weight and the optimal pH of this lipase were respectively about 60 kDa and 4.5. It appeared that this lipase shared conformational homology with the *Rhizomucor miehei* lipase.

In more recent works, two proteins were purified from oil body proteins of *Brassica napus* cultivars. Submitted to a proteomic analysis, they showed high similarities to the lipase/hydrolase family. These similarities suggest that these two proteins could be involved in oil body degradation (Katavic et al., 2006).

Lipases belong to the α/β hydrolase fold family. The active site of many α/β hydrolases is composed of a catalytic triad formed by a nucleophile Ser, Cys or Asp residue which is followed by glutamate (Glu) or aspartate (Asp) and a histidine (His) (Ollis et al., 1992; Cygler et al., 1993; Holmquist, 2000).

These structural and functional features common to

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lipases belonging to different species, suggest that this homology might be widely shared by other lipases and related enzymes and could be used to investigate and purify new lipase activities.

In this study, we isolated a true lipolytic activity at pH 7.5 from germinating sunflower (*H. annuus* L.) seedlings associated to oleosomes. Two bands of about 61 and 66 kDa were isolated from the active lipase fraction after oleosomes extraction and a diethylaminoethyl (DEAE) exchange chromatography coupled with hydroxyapatite column. Polyclonal antibodies (PAbs) were produced against each protein bands, P-61 and P-66 and the specificity against pancreatic lipase was determined.

MATERIALS AND METHODS

Isolation of oleosomes

The cotyledons from sunflower (*Helianthus annuus* L. var Albena from CETIOM, Paris) of 3-day-old sterilized seedlings were used. Seeds were soaked in running water for 12 hours and germinated in darkness at 30°C. After oleosomes extraction using Qu et al. (1986) method adapted to the sunflower, the washed lipid bodies were extracted with diethyl ether to remove the triacylglycerols. The suspension was centrifuged at 100 000 g during 90 min and the resulting supernatant was precipitated with 52 % ammonium sulphate before 90 min of dialysis (Bahri, 2000) against buffer A: Tris-HCl 20mM, 1mM Dithiothreitol (DTT), 1mM Benzamidine pH 7.5, then loaded onto a DEAE trisacryl M column exchange chromatography (2x20 cm) coupled to hydroxyapatite column (8x5 cm) (Oursel et al., 1990). The columns have been previously equilibrated with the elution buffer A, then washed with a linear gradient of 0 to 0.8 M NaCl in the same buffer. All the procedure was performed at 4°C.

Lipase activity

Lipase activity was measured at pH 7.5 by a colorimetric method adapted from Duncombe (1962) and Nixon and Chan (1979). The fatty acids released were converted into copper soaps. The substrate (sunflower oil) was purified according to Hulanicka et al. (1964) then emulsified with gum arabic (10%). The reaction was carried out at 30°C for 20 min in a shaker water bath at 30°C. All assays were performed in triplicate.

SDS-PAGE analysis

Proteins were separated by SDS PAGE according to Laemmli (1970) on a 10% acrylamide resolving gel and a 5% acrylamide stacking gel; then they were silver stained. Their molecular weights were determined relatively to marker proteins: phosphorylase A (Mr 100 kDa), SAB (Mr 66 kDa), ovalbumin (Mr 45 kDa), carbonic anhydrase (Mr 30 kDa), trypsin inhibitor (Mr 20 kDa) and cytochrome C (Mr 12.38 kDa).

Antibody preparation

We prepared Pabs raised against each one of the two bands (66 and 61 kDa) of the active lipase fraction as described in the following procedure: after a preparative electrophoresis (SDS PAGE 10%) according to Laemmli (1970), the 66 (P-66) and 61 kDa (P-61) bands were extracted from the gel. Each band was injected subcutaneously to a rabbit in emulsion in the presence of 200 µl of Freund's incomplete adjuvant (Sigma). Two intramuscular injections were given six weeks later with 200 µg of each antigen in Freund's complete adjuvant into the corres-ponding rabbit. A blood sample was taken and the immune responses were tested in the presence of the oleosomal extract. A month later, a second booster was purchased, in the same conditions as the second injection. Rabbits were bled two weeks later and IgG were purified through a DEAE cellulose chromato-graphy column.

Enzyme linked immunoadsorbent assay (ELISA)

Binding of oleosomal extract to rabbit antiserum

Different dilutions of the oleosomal extract (from 2 to 5 µg) in coating solution (sodium carbonate, pH 9.6) were added to appropriate wells of a microtitration plate (Nunc). The plate was incubated overnight at 4°C then washed twice with phosphate buffer saline (PBS)-Tween 20. Rabbit antiserum directed against lipolytic fraction, diluted at (1: 500) was added to the corresponding wells and incubated 2 h at room temperature. The washing procedure with PBS and PBS-Tween 20 was then applied before adding the 1: 1000 dilution of peroxydase - conjugated goat anti-rabbit antibody (Sigma). The peroxydase substrate, orthopheny-lenediamine dichloride (OPD) was added and absorbance was measured at 492 nm. Negative controls respectively used were invertase and normal rabbit serum.

The porcine pancreatic lipase inhibition

The binding inhibition of antibodies directed against the P-61 sunflower protein to the homologous antigen (2 µg of sunflower oleosomal extract coated by well), was performed by indirect ELISA using a chosen immune serum dilution (1:500) and the porcine pancreatic lipase (Fluka) was used as inhibitor in different concentrations ranging from 12.5 to 200 µg /ml, in PBS buffer.

Western blotting

Oleosomal membrane proteins separated with SDS-PAGE were transferred onto nitrocellulose membrane by electroblotting for 2 h at 1 A (Towbine et al., 1979). The membrane was blocked by incubation overnight in 3% Bovine Serum Albumin (BSA) in PBS buffer and washed with PBS-Tween 20 (0.1%). The membrane was then cut and separated sheets were respectively incubated during 2 h (37°C) with different dilutions of the two immunsera. After washing with PBS Tween 20, the membranes were incubated for 2 h with 1:1000 dilution of peroxidase–conjugated goat anti–rabbit antibody (Sigma), washed with PBS-Tween 20, and then incubated with the substrate: 3, 3'-diaminobenzidine was added with hydrogene peroxide (H_2O_2). Finally, the reaction was stopped after 20 min, with water.

Protein determination

At each step, protein quantification was determined according to Bradford (1976). BSA was used as standard.

RESULTS

Evidence of a true lipase activity in sunflower oleosomes

Starting with 3-days-old sunflower (*H. annuus* L.)

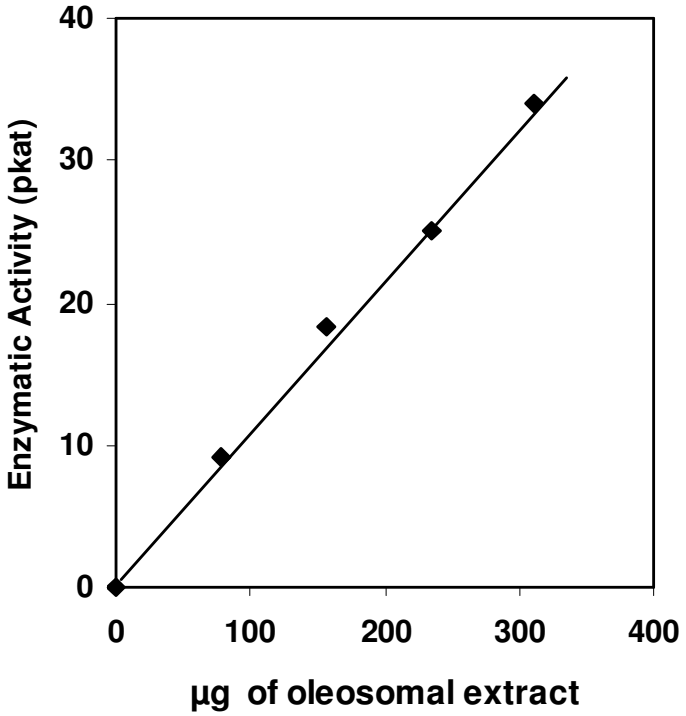

Figure 1. Enzymatic activity of the delipidated oleosomal extract.

germinating seedlings, we used a purification procedure as indicated above that allowed us to obtain an oleosomal preparation enriched with lipolytic activity. True lipase activity was found in unbound hydroxyapatite column fractions. As shown in Figure 1, by using purified sunflower oil (50 mM) as substrate, we observed the presence of a true neutral lipase activity in sunflower seedlings oleosomes extracts. However, the detected activity in this fraction was found low.

The effect of pH on the hydrolysis of the substrate was measured. Two optimal pH of 4 and 7.5 were identified (Figure 2) with respectively specific activities of about 90 and 200 pkat/mg. Neutral value (pH 7.5) was retained for ulterior tests, for the purpose of investigating the capacity of the enzyme to hydrolyse TAG under conditions usually found *in vivo*.

Silver stained SDS-PAGE from the active oleosomal preparation showed two protein bands respectively of 61 and 66 kDa (Figure 3).

The 61 (P-61) and 66 kDa (P-66) protein bands were extracted from the gel and injected into rabbits. Serums from rabbits were collected and specificity of antibodies was determined. Indirect ELISA was performed to test the specificity of the corresponding serums by incubating 5 µg of delipidated oleosomal extracts from 3-days-old germinating seedlings in the presence of 100 µl of different dilutions from each antiserum (1:1000 to 1:125). PAbs were only produced against the P-61 band. It seems that the upper band (P-66) was not immunogenic to the rabbit (figure 4A).

The PAbs prepared against the P-61 band showed by Western Blotting, a specific reaction to the homologous antigen, using total oleosomal extract (Figures 4A and 4B). The antibodies produced against the P-61 reacted neither with the P-66 nor with other proteins of sunflower oleosomal extracts (figure 4B).

Our results are in agreement with antibodies specificity to the P-61 protein. These results also suggest that the bands P-61 and P-66 do not share any common determinant and could correspond to different proteins.

The anti P-61 antibodies inhibit plant and porcine lipase activities

In order to test the last hypothesis, we measured the enzyme activity by incubating the oleosomal extract in the presence of different dilutions of anti-P-61 antiserum. We observed a decrease in the lipase activity which was 100% inhibited in the presence of 1:100 diluted immune serum (Figure 4C), indicating that the P-61 band carries alone all the lipase activity present in the oleosomal protein extract.

As expected, antiserum against P-66 had no effect on the lipase activity. The corresponding results were analogous to those of the controls using normal rabbit serum.

Since pure lipases from animals are available contrarily to plant lipases, we tested the anti-P-61 immune serum against a pure pancreatic lipase from hog (Fluka), a

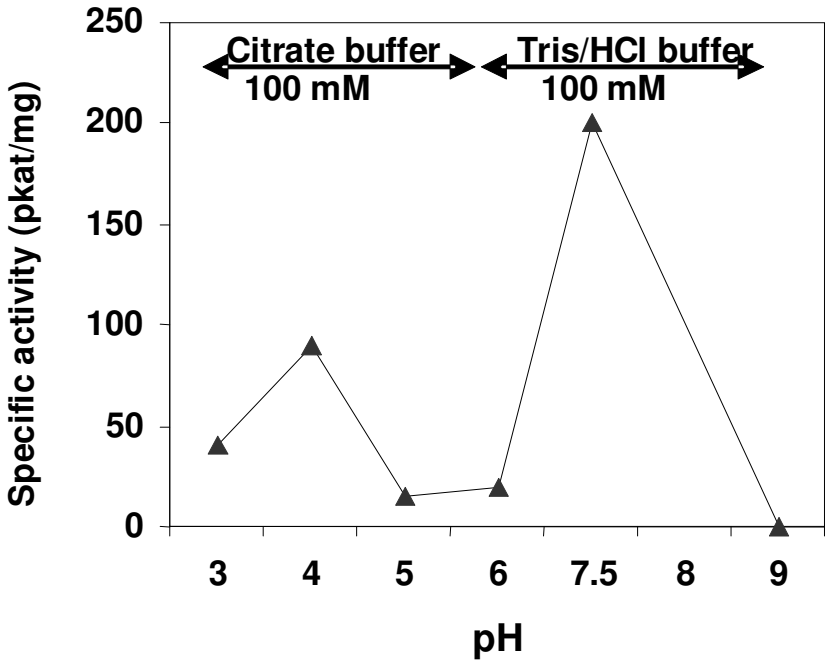

Figure 2. Effect of pH on the rate of lipolysis by the oleosomal lipase.

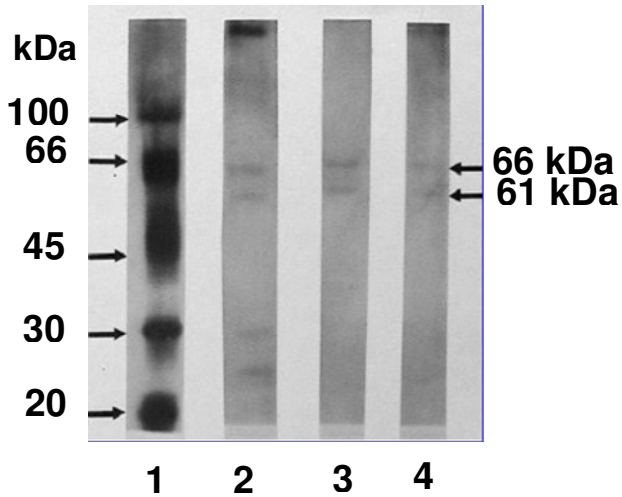

Figure 3. SDS-polyacrylamide gel electrophoresis profile of delipidated sunflower oil bodies extract. Aliquots from dialysis extract, unbound DEAE column and unbound HA column fractions were analysed. The gel was silver-stained. Position and relative mass (kDa) of molecular markers are given in the left margin. Lane 1, molecular weight markers; lane 2, dialysis extract; lane 3, unbound HA; lane 4, unbound DEAE fractions. The arrows at the right of the gel indicate the location of the co-purified proteic bands corresponding to the lipolytic activity. Molecular weight markers: phosphorylase A (Mr 100 kDa), SAB (Mr 66 kDa), ovalbumin (Mr 45 kDa), carbonic anhydrase (Mr 30 kDa), trypsin Inhibitor (Mr 20 kDa).

protein that is presumed to be different from plant lipases. We observed the decrease of the porcine enzyme activity reaching 100% inhibition with a 1: 100 anti-P-61 immune serum dilution. This inhibition is specific since rabbit normal serum used at the same dilutions as control does not inhibit the porcine lipase activity (Figure 5A). This result indicates that the PAbs to sunflower P-61 specifically recognized the porcine lipase and were able to inhibit totally the activity of the pure pancreatic lipase.

Cross reactivity with porcine pancreatic lipase

In order to confirm the cross-reaction with porcine lipase, we performed immunoblotting using PAbs - P-61 protein tested on a 50 kDa pure porcine lipase by SDS PAGE (Figure 5B). The polyclonal anti-P-61 antiserum recognized specifically the porcine pancreatic lipase. A specific band appeared at a molecular weight of 50 kDa. In order to perform an indirect ELISA inhibition test, we purified IgG from the rabbit immune serum raised against P-61. We used different dilutions of pure porcine pancreatic lipase as inhibitor of rabbit IgG binding to P-61 antigen (Figure 5B). The inhibition reached 60%, suggesting the existence of a high cross-reactivity between porcine and sunflower lipases (Figure 5C). These results indicate that cross-reactivity would be the result of structural homology that involves the active sites of both enzymes.

DISCUSSION

Difficulties that have been encountered by several

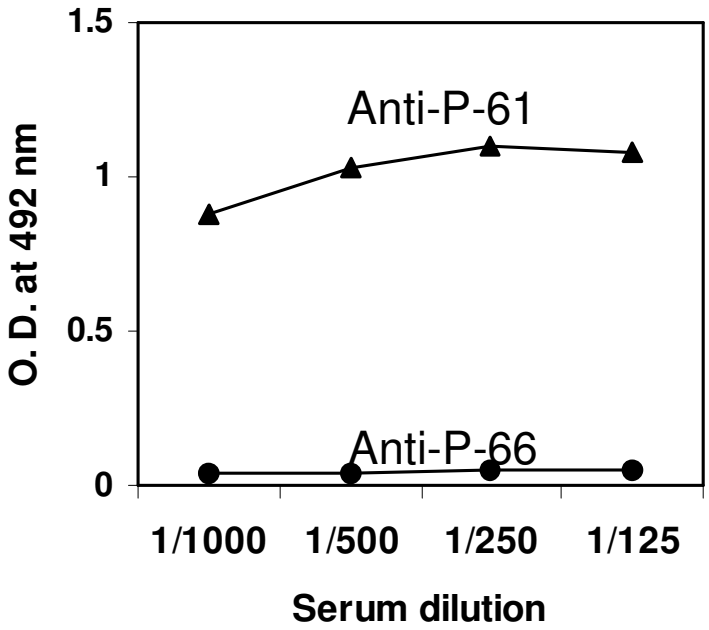

Figure 4A. Specific recognition between polyclonal antibodies raised against sunflower P-61 and the homologous antigen; ELISA immune serum anti P-61 specific response to the sunflower oleosomal extract. ◆ No change was observed in the amount of antibodies raised against sunflower P-66 incubated with the total oleosomal extract. The curve was superposed to the control.

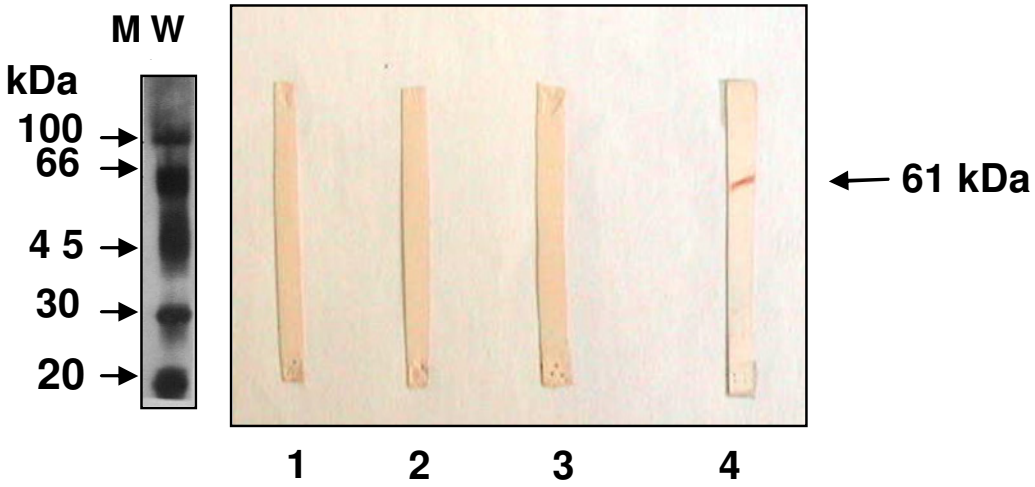

Figure 4B. Specific recognition between polyclonal antibodies raised against sunflower P-61 and the homologous antigen; immunoblotting of the sunflower oleosomal extract separated by SDS-PAGE. Lane 1, P-61 control; lane 2, P-66 control; lane 3, total oleosomal extract incubated with the immune serum raised against P-66; lane 4, total oleosomal extract incubated with the immune serum raised against P-61. The arrow at the right of the nitrocellulose membranes indicates the location of the oleosomal lipase specifically recognized at 61 kDa.

authors to purify and characterize plant lipases are due to their low activity *in vitro* and to the catalytic mechanism that does not follow the classical Michaelis-Menten model, but requires a three dimensional catalysis including an interfacial adsorption and activation (Verger, 1997).

To study the oleosomal sunflower lipase that has not been identified yet, we started from oleosomes,

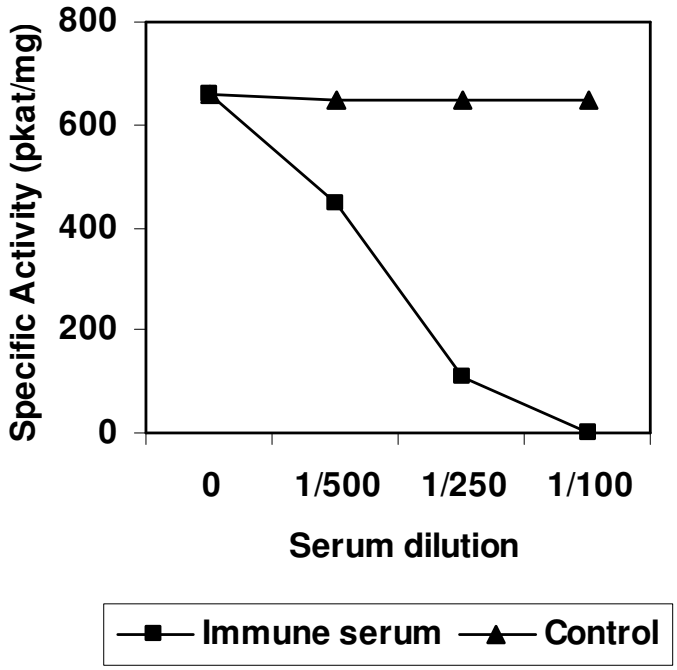

Figure 4C. Specific recognition between polyclonal antibodies raised against sunflower P-61 and the homologous antigen. Immunoprecipitation of sunflower lipase by the immune serum directed against P-61. ■ Lipase activity as determined by measurement of free fatty acid release using a copper soap as described in the experimental procedures.

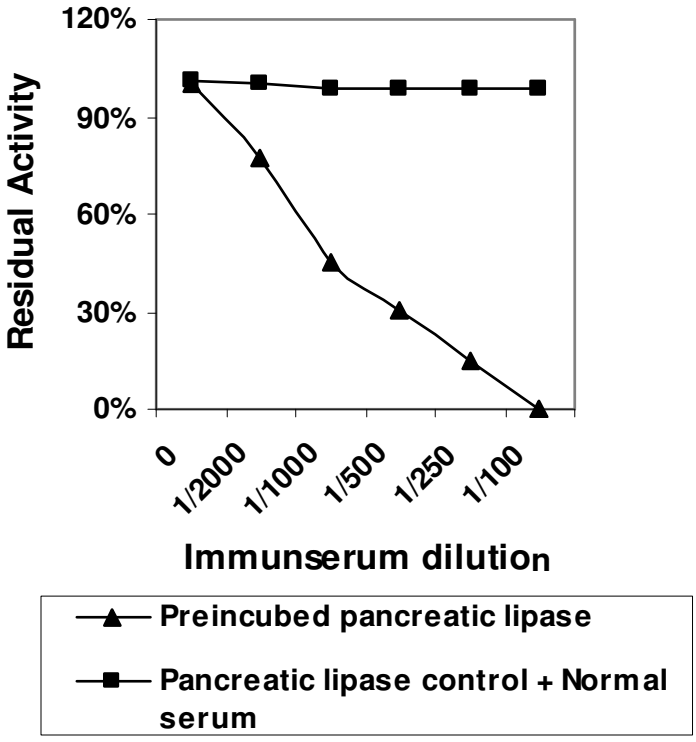

Figure 5A. Cross-reactivity between porcine pancreatic lipase and P-61 oleosomal proteic band. Inhibition of porcine pancreatic lipase activity by sunflower anti-P-61serum.

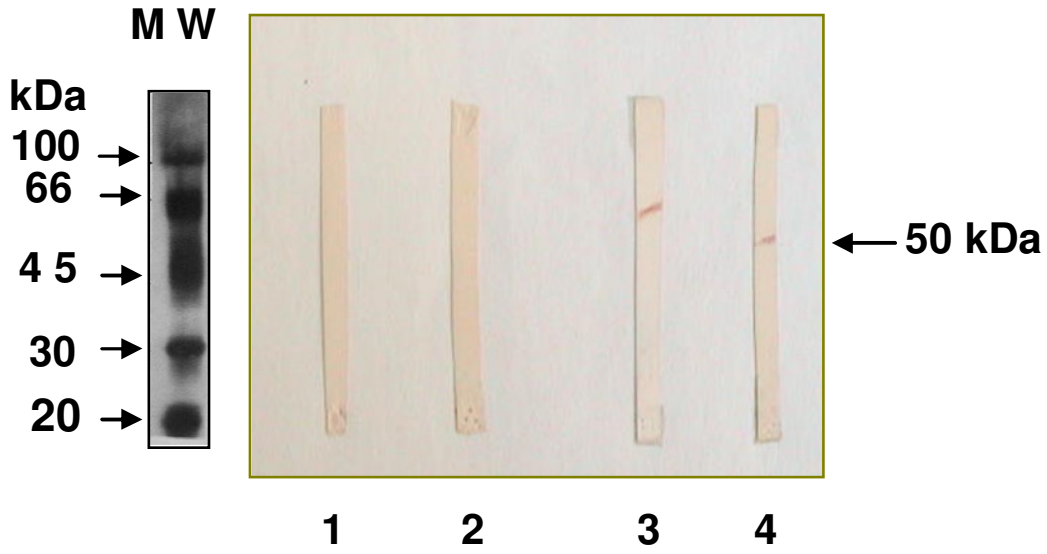

Figure 5B. Cross-reactivity between porcine pancreatic lipase and P-61 oleosomal proteic band. Western Blotting of pure porcine lipase separated by SDS PAGE showing cross- reactivity with anti-P-61 sunflower oleosomal extract immune serum. Lane 1, porcine pancreatic lipase control; lane 2, porcine pancreatic lipase control; lane 3, sunflower oleosomal lipasic activity recognized at 61 kDa by the anti-P-61 serum; lane 4, porcine pancreatic lipase incubation in the presence of the immune serum raised against P-61 oleosomal sunflower proteic fraction. The arrow at the right of the figure indicates the location of the pure porcine pancreatic lipase specifically recognized at 50 kDa.

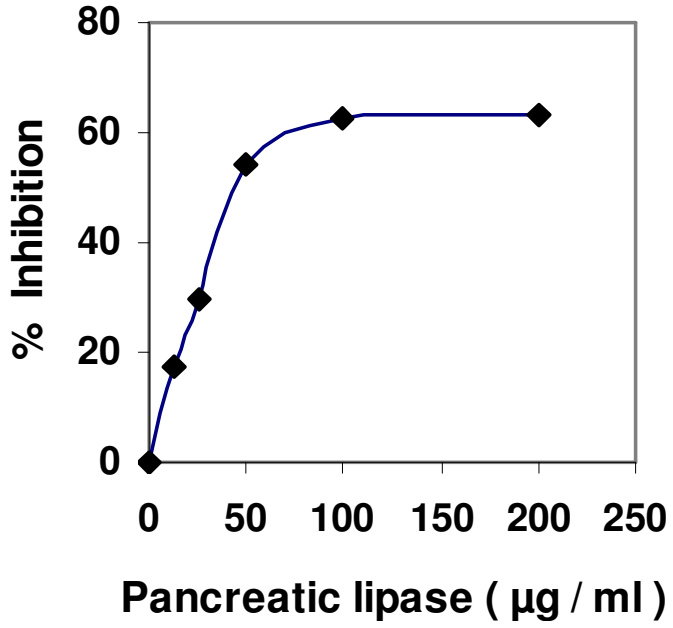

Figure 5C. Inhibition of the binding by ELISA of antibodies raised against P-61 sunflower oleosomal extract to the homologous antigen using as inhibitor porcine pancreatic lipase at different concentrations. Polyclonal antibodies dilution (1: 500).

presuming that lipase would be associated with these TAG stocking organelles. Purified sunflower oil used as substrate reveals the presence of a true lipase activity. The latter was detected at pH 7.5 in a preparation containing two protein bands of respectively 61 and 66 kDa. P-61 and P-66 do not share any common

determinant. This conclusion excludes the hypothesis that the two co-purified protein bands would derive from each other but our result does not exclude that these two proteins would interact with each other.

Our study shows that sunflower lipase activity is carried by a protein with about 61 kDa molecular weight, a size analogous to that of other plant lipases as described by Lin and Huang (1984), Maeshima and Beevers (1985) and Eastmond (2004). This activity is however weakly expressed in the oleosomal extract (Bahri, 2000) as compared to animals and microorganisms lipases.

We also put in evidence, the usefulness of the immunochemical approach (Lin and Huang, 1984; Maeshima and Beevers, 1985; Murphy et al., 1989) in the study of plant lipases. PAbs against P- 61 kDa protein have been shown to be specific to the P-61 sunflower protein band. They inhibit entirely the lipase activity detected in the enzymatic extract. They also inhibit totally (100%) the activity of a pure porcine pancreatic lipase. This lipase inhibits at 60% the binding of the PAbs to their homologous plant antigen. Taken together, these results carry out the existence of a cross reactivity between the 50 kDa porcine and P-61 sunflower proteins. This cross-reactivity would be the result of structural homology that involves the active sites of both enzymes, probably the consensus sequence Gly-X-Ser-X-Gly described in diverse species (Antonian, 1988).

This result strongly suggests that P-61 band recognized by the immune serum could contain a true lipase.

This study has also been extended to pure lipases from *Candida rugosa* and *Rhizomucor miehei*. We noted that P-61 Pabs specifically recognized the fungal lipases, by ELISA and western blotting tests, indicating the cross reactivity between sunflower P-61 and the pure micro-organic lipases (data not shown).

Indeed, the porcine and fungal 3-D lipase structures have also been elucidated (Brady et al., 1990; Brozowzski et al., 1991; Grochulski et al., 1993). They all belong to the α/βhydrolase fold family that exhibits characteristic features which include a Ser-Acid-His catalytic triad with a conserved motif GXSXG surrounding the nucleophile Ser residue (Ollis et al., 1992; Cygler et al., 1993; Holmquist, 2000; Bell, 2002).

A bioinformatic approach has shown for plants the presence of a functional domain corresponding to the catalytic triad IPR000734. By using Interpro basis (www.interoro.com), we observed that only two species: virdiplantes (*Picea sitchensis* AC accoding Interpro: 33090) and *streptophyta* (AC accoding Interpro: 35439) have shown the consensus sequence of the catalytic triad. This domain is highly conserved and kept in several species. However, these domain annotations are putative; inferred by electronic assay. No gene ontology corresponds to these not yet investigated domains.

On the basis of the immunochemical study, we suggest that structural homology between sunflower lipase porcine and fungal lipases, involves the active sites of these molecules that share a highly conserved homologous structure which would concern rather conformation than the primary structure. That conformation would involve the α/β hydrolase fold and at least the His residue present in the catalytic triad (Schrag, 1991; Dodson et al., 1992; Nardini and Dijkstra, 1999).

The use of the immunochemical strategy could be a successful tool in the investigation of plant lipases. Further experiments like mass spectrometry analysis will help confirm the conserved structural similarity between plant, animal and microorganism lipases.

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