Arab Gulf J. Scient. Res., **16** (2), pp. 347-358 (1998)

Properties and Purification of 42 KDa Lipase Subunit from Germinating Linseeds

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ABSTRACT. Electrophoretic analysis of the germinating linseed proteins shows alterations in a protein with a molecular weight of 42 KDa. This protein accumulates after 36 hours of germination in synchronization with an increase in lipase activity, and a decrease in the quantity of the total lipids. The 42 KDa subunit was found to be a lipid body membrane protein. This protein was isolated and identified by immunoprecipitating technique as a subunit of lipase. The linseed lipase reacted with a wide range of triacylglycerols and had optimal activity at pH 4.7. The activity of the enzyme was slightly affected by high concentration of salts and EDTA, while high concentrations of non-ionic detergents exhibited a pronounced inhibitory effect. These data suggest that the isolated 42 KDa protein is most likely a linseed acid lipase responsible for the breakdown of lipids during germination.

Seeds of some plants store triacylglycerols (TAG) as small discrete intracellular organelles called oil bodies (Yatsu and Jacks 1972, Huang 1985, Stymme and Stobart 1987, Huang *et al.* 1991, Siedow 1991, Tzen *et al.* 1993). These oil bodies are used as food reserves for germination and post germination growth of the seedling.

Lipase; (triacylglycerol acylhydrolase, EC 3.1.13) is the enzyme catalyzing the breakdown of the TAG into glycerol and free fatty acids (Hammer and Murphy

Key Words: Acid lipase; Triton X-100-solubilized lipid body membrane proteins (XLBP); ether-extracted lipid body membrane proteins (ELBP).

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1993). This enzyme has been purified to homogeneity in only three species; the lipid body neutral lipase from the scutella of corn (Lin and Huang 1984), the glyoxysomal alkaline lipase from castor bean (Maeshima and Beevers 1985) and the major lipase in the megagametophyte of pinyon (*Pinus edulis* Engelm). (Hammer and Murphy 1993). The corn, castor bean and *Pinus edulis* lipases have a protein size of 65, 62 and 64 KDa respectively.

The present study reports on the properties and purification of 42 KDa linseed lipase subunit and its relation to TAG degradation.

Materials and Methods

Plant material

Linseed (*Linum usitatissimum* L., var. Giza 5) obtained from the Agricultural Research Center, El-Dokki, Giza, Egypt, were surface sterilized with 70% ethanol for 3 min. After rinsing thoroughly with distilled water, the seeds were transferred to petri-dishes containing 6 ml distilled water per gram dry weight of the seeds and germinated at room temperature (23° C) in the dark (Lin and Huang 1984). Seeds were harvested every 12 hour for consecutive 5 days, during which period the seed coat was removed and a portion was freeze-dried.

Initial localization study

After removing seed coats, a portion of the germinated seeds were washed with distilled water, macerated in ice-cold grinding buffer (consisted of 0.4 M sucrose, 10 mM KCl, 2 mM EDTA, 2 mM dithiothreitol (DTT), 1 mM $MgCl_2$ and 165 mM tricine-NaOH buffer (pH 7.5)) and filtered. Following centrifugation at 1300 g for 10 min at 5° C, the supernatant was removed and centrifuged at 12000 g for 30 min at 5° C. Fifty-ul samples of the upper lipid pad, the supernatant and the grinding buffer-suspended final pellet from the second centrifugation were assayed colorimetrically for lipase activity (Maeshima and Beevers 1985).

Enzyme assay

Linseed lipase activity was assayed colorimetrically for the initial localization, gel permeation, pH optima and TAG substrate specificity studies (Hammer and Murphy 1993, Huang 1985). In a Teflon screw-top glass tube, 100 ul of the enzyme fraction and 100 ul substrate (50 mM trilinolein) suspended in 5% gum acacia by mixing for 30 s with Tekmar tissuemizer (Tekmar, Cincinnati, OH, USA) were added to 800 ul assay buffer (100 mM succinate-NaOH, pH 4.7, 5 mM DDT) and incubated for 30 min at 25° C. For pH effects on activity, an assay buffer containing

either 100 mM citric acid-citrate, Tris-malate, or glycine and 5 mM DDT was used. The reaction was stopped by heating the tube at 100° C for 5 min. Fatty acids released in the reaction mixture were quantified using the colorimetric method of Huang (1985) with a standard curve obtained with linoleic acid. Activity was expressed in nmol fatty acids cleaved min^{-1} mg $protein^{-1}$. Controls consisted of reaction mixtures with heat-denatured enzyme and controls without substrate.

A flourometric lipase assay, described by Haung (1985), was used in the immunoprecipitation and reagent effect studies.

Preparation of lipid body membrane proteins

Approximately 50 g of the germinating seeds were ground in a Waring blender with 50 ml grinding buffer (as above). The homogenate was filtered through Miracloth. Each 10 ml of the filtered crude homogenate was placed in a 38.5 ml centrifuge tube and overlaid with grinding buffer containing 0.2 M sucrose to almost fill the tube. The tubes were centrifuge at 10 000 g for 15 min at 5° C. The resulting lipid pad was resuspended in 10 ml of 0.4 M sucrose grinding buffer until the tube is almost full and centrifuged again as above (Murphy and Cummins 1990, Hammer and Murphy 1993). The resulting pad contained the washed, isolated lipid bodies.

For electrophoretic analysis, the lipid pad containing the washed, isolated lipid bodies was placed in a 50-ml screw top tube with 20 ml detergent-containing buffer medium (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1% Triton X-100) and orbitally shaken for 3 h at 5° C. After that the suspension was centrifuged at 50 000 g for 15 min and the centrifuge tubes were carefully placed upright in a –80° C freezer. After ca 16 h, the lipid pad was completely scraped off the frozen supernatant which contained the Triton X-100-solubilized lipid body membrane proteins (XLBP) (Hammer and Murphy 1993). For immunoprecipitation study, the lipid pad containing the washed, isolated lipid bodies were resuspended in 20 ml sucrose-containing buffered medium (20 mM Tris-HCl pH 7.5, 1 mM DDT, 0.2 M sucrose) and extracted 5 times with a double volume of diethyl ether to remove the triacyglycerols. Diethyl remaining in the final aqueous fraction was evaporated with stream of N_2. The aqueous fraction was then centrifuged at 100 000 g for 90 min (Lin and Huang 1984) with the resulting supernatant being the ether-extracted lipid body membrane proteins (ELBP). XLBP and ELBP had the same distribution of proteins when visualized using SDS-PAGE (Fig. 4).

Gel permeation chromatography

Twenty-five ml of ELBP (750 ug protein ml^{-1}) was incubated with 1% Triton X-100 for 1 h at 5° C, then concentrated to 4.6 ml using a Centriprep 30 concentrator

(Amicon, Danvers, MA, USA). The concentrate was applied to a Sephacryl S-300 (Pharmacia. Uppsala, Sweden) gel permeation column (2.6 x 90 cm) and eluted with detergent-containing buffered medium at 0.5 ml min^{-1}. After 5 h (Vo = 163 ml), 5 ml fractions were collected for 6 h (Lin and Huang 1984). Fractions were assayed colorimetrically for protein and lipase activity.

Purification of 42 KDa protein

The 42 KDa protein from linseed proteins was purified to homogeneity by the protocol used by Hammer and Murphy (1993).

Protein determination

Protein was measured by the dye-binding technique (Bradford 1976).

Gel electrophoresis

The seed meal proteins were extracted with 0.125 M Tris/borate buffer, pH 8.9, containing 2% SDS and then electrophoretically resolved in 12% polyacrylamide gel following the method of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R-250.

The gel was scanned in LKB Recording Laser Densitometer equipped with LKB 2220 Recording Integrator to quantify the concentration of the 42 KDa protein.

Estimation of total lipids and fatty acid composition

Total lipids were extracted and methylated according to Folch *et al.* (1957) and Luddy *et al.* (1968). The methylated fatty acids were estimated in a Hewlett Packard (Model No. 5730A) GLC.

Antibody preparation

Antibody of 42 KDa protein was prepared in rabbit according to the modified method of Hammer and Murphy (1993).

"Western" blotting technique

This technique was carried out according to the method published by Towbin *et al.* (1979).

Results and Discussion

Experiments with lipid pad showed optimal activity for linseed lipase at acidic pH 4.7 and was particularly active in the period between 36 and 84 hours of

germination (Fig. 1). On the other hand, the pellet and soluble fractions possessed a slight basal linseed lipase activity. These data agree well with the work of Hammer and Murphy (1993) on *Pinus edulis.*

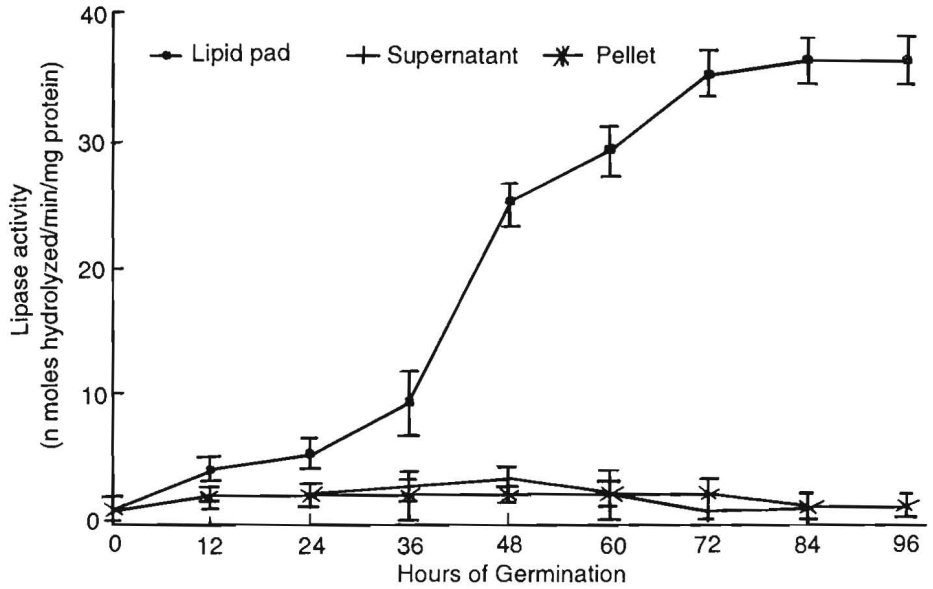

Fig. 1. Acid lipase activity of lipid pad, supernatant and pellet from dry (hour 0) and germinating (hours 12-96) linseed calculated as using the protein concentration of each fraction.

Lipids were extracted from dry and germinating seeds at intervals and their fatty acid composition were analyzed. The data in Fig. 2 show that the fatty acids of dry seeds are; palmitic, stearic, oleic, linoleic, linolenic. The fatty acids follow the same pattern of variation as lipids and their degradation during germination were almost similar except for linolenic acid which represents the majority of linseed lipids (Fig. 2).

Lipids degradation was accompanied by accumulation of a protein with molecular weight of 42 KDa (Fig. 3A). Densitometer scans of the tracks in Fig. 2A show a 42 KDa protein that accumulated at 36 hours of germination and reachs a maximum accumulation at 84 hours of germination (Fig. 3B). The accumulation of 42 KDa subunit at 36 hours of germination and its resistance to degradation throughout the coruse of germination (Fig. 3A and B) in combination with: 1) the increase in enzyme activity (Fig. 1), and 2) the sharp decrease in lipids and linolenic acid (Fig. 2) suggest that the 42 KDa protein could be the linseed lipase and encourage us to purify this subunit and to study its functional properties.

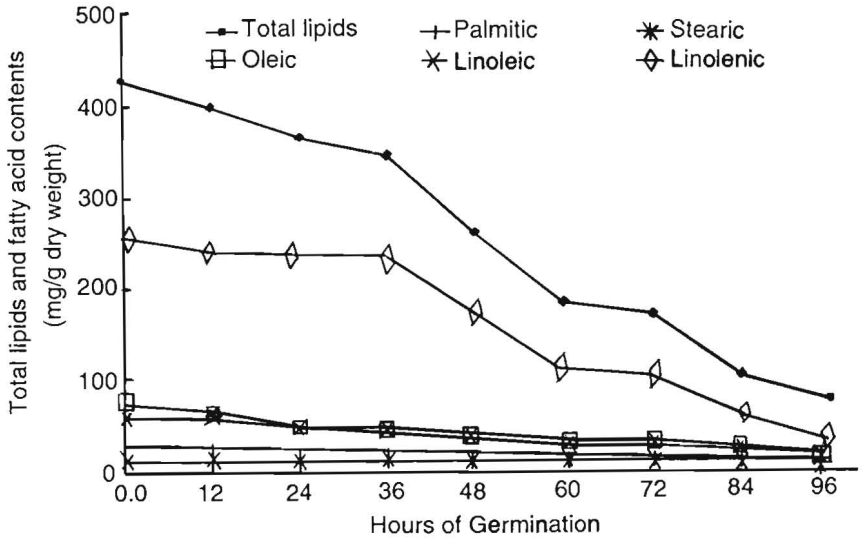

Fig. 2. The concentrations of linseed lipids and fatty acids in dry and germinating seeds.

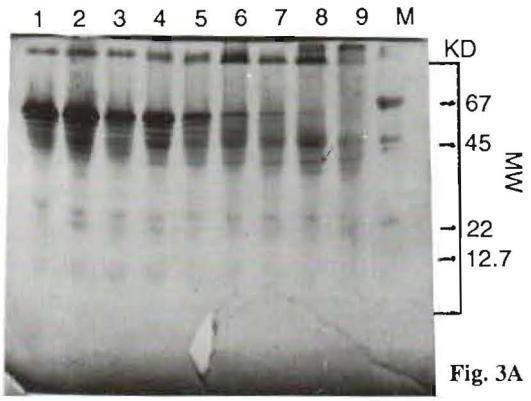

Fig. 3A

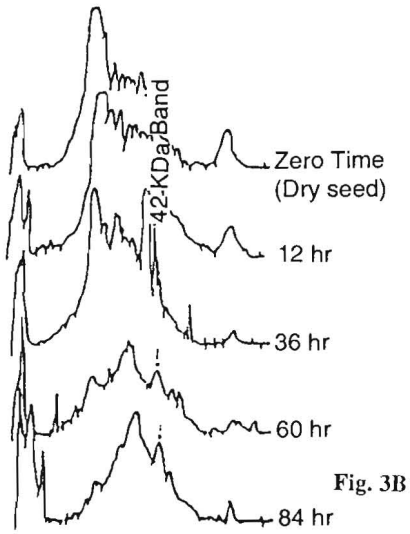

Fig. 3B

Fig. 3A. SDS electrophoretic patterns of germinating linseed. Lane M, mol. wt. markers consisting of BSA (67 KD), Ova albumin (45 KD), trypsin inhibtor (22 KD) and cytochrome-C (12.3 KD); Lane 1, mature linseed prior to germination; lane 2-9, after 12 to 96 hours.

Fig. 3B. Scans of gel patterns of germinating linseed. A mature seed prior to germination (Dry seed); B, after 24 hr; C, after 48 hr; D, after 72 hr; E, after 96 hr.

When XLBP were separated on Sephacryl S-300 gel permeation column, they exhibited an apparent molecular weight of 190 000. Further purifications using ion exchange chromatographogy and hydrophobic interaction chromatography were failed; as in *Pinus edulis* (Hammer and Murphy 1993). The failure was apparanetly due to that the enzyme did not elute with solvents that would retain activity with or without non-ionic detergent. Thus, further attempts to purify and identify linseed lipase were made through immunological techniques.

As mentioned above, the molecular weight of the linseed lipid body acid lipase eluted from Sephacryl S-300 gel permeation column was 190 000. This molecular weight was about fourfold the subunit molecular weight. Thus, the subunit structure of linseed lipid body acid lipase agrees well with subunit structure of lipases extraced from other species (Maeshima and Beevers 1985, Hammer and Murphy 1993). The similarity in subunit structure paralleled the increase in the amount of 42 KDa protein and the rise in lipase activity during germination (Figs. 1 and 2). For these reasons, the 42 KDa was isolated using preparative SDS-PAGE.

The purification fractions, whose proteins components were separated using SDS-PAGE, are shown in Fig. 4. Crude cotyledons extract from seed germinated 84 hours (lane 1) was used as a source for the preparation of isolated lipid bodies (Fig. 4, lane 2), and the lipid body membrane proteins were solubilized in a 1% Trition X-100 buffer (XLBP, Fig. 4, lane 3). The XLBP were separated using preparative SDS-PAGE and the 42 KDa protein was isolated by electroblotting from an excised gel slice (Fig. 4 lane 4). Antibodies of the 42 KDa protein were highly specific as shown by a Western blot of the SDS-PAGE separated XLBP (Fig. 4, lane 5). The appearance of minor bands of molecular weight less than 42 KDa in the Western blot suggest that the antibody is reacting to breakdown products of the 42 KDa protein or that there is some degree of antigenic similarity among several lipid body membrane proteins in denaturated state.

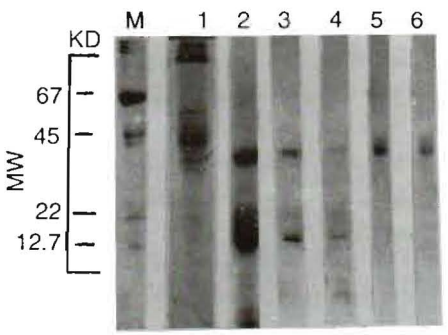

Fig. 4. SDS-PAGE (lanes M and 1-4) and Western blot (lane 5) of hour 84 germinating linseed. Lane M, marker proteins; Lane 1, seed meal extract of hour 84 germinating linseed; lane 2, lipid pad; Lane 3 and 6, XLBP; Lane 4, ELBP; Lane 5, isolated 43 KDa lipase subunit. The Western blot was probed with rabbit antibodies against the purified 42 KDa protein of germinating linseed.

Immunoprecipitation, using ELBP, indicated that the anti-42 KDa protein was able to precipitate acid lipase activity from the reaction mixture (Fig. 5), indicating that the antibody recognized the native lipase enzyme. Therefore, the 42 KDa protein appears to be a subunit of lipase enzyme.

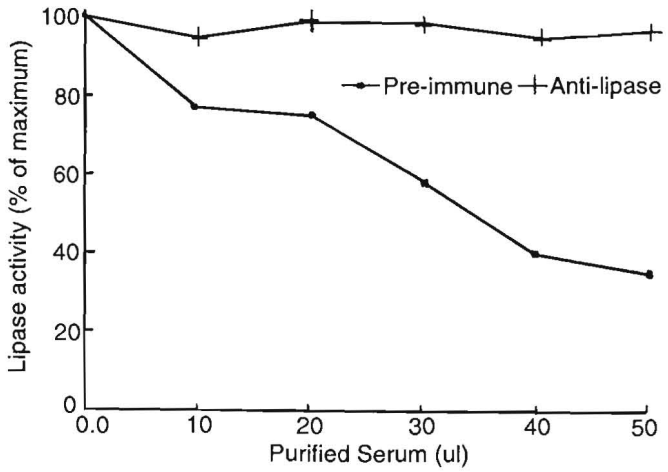

Fig. 5. Immunoprecipitation of linseed lipid body lipase by purified pre-immune and anti-42 KDa liapse subunit IgG from rabbit. Lipase was from ELBP. Lipase was assayed fluorometrically.

Using ELBP, pH optimum for colorimetric lipase reaction was between pH 4.5 and 4.7 (Fig. 6). The pH dependence of colorimetry activity matched that for the lipid body lipase of castor bean (measured by titration) and *Pinus edulis* (measured colorimetrically) (Hammer and Murphy 1993, Ory 1969).

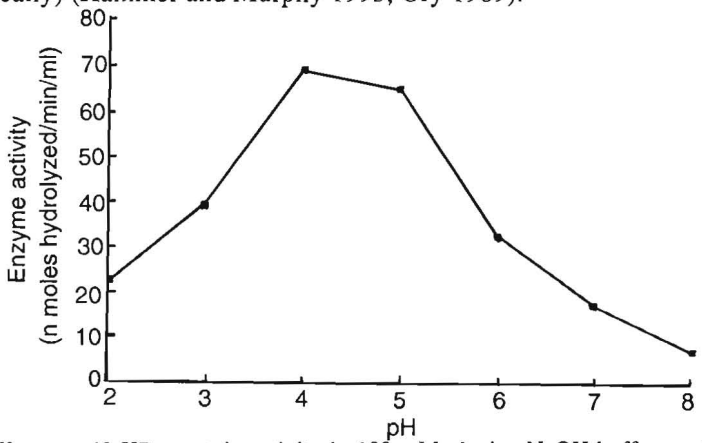

Fig. 6. pH effects on 42 KDa protein activity in 100 mM glycine-NaOH buffer containing 5 mM DTT.

Linseed lipase was assayed for enzyme activity, using a wide range of triacylglycerols (TAG). The highest activity was on C18:n sidechain group (Fig. 7). These data also showed that linseed lipase did not hydrolyze mono- and diglycerides. Linseed lipid body lipase was similar in lack of specificity to the lipid body lipases of rape seed and *Pinus edulis* (Hammer and Murphy 1993, Hills and Murphy 1988, Lin *et al.* 1986).

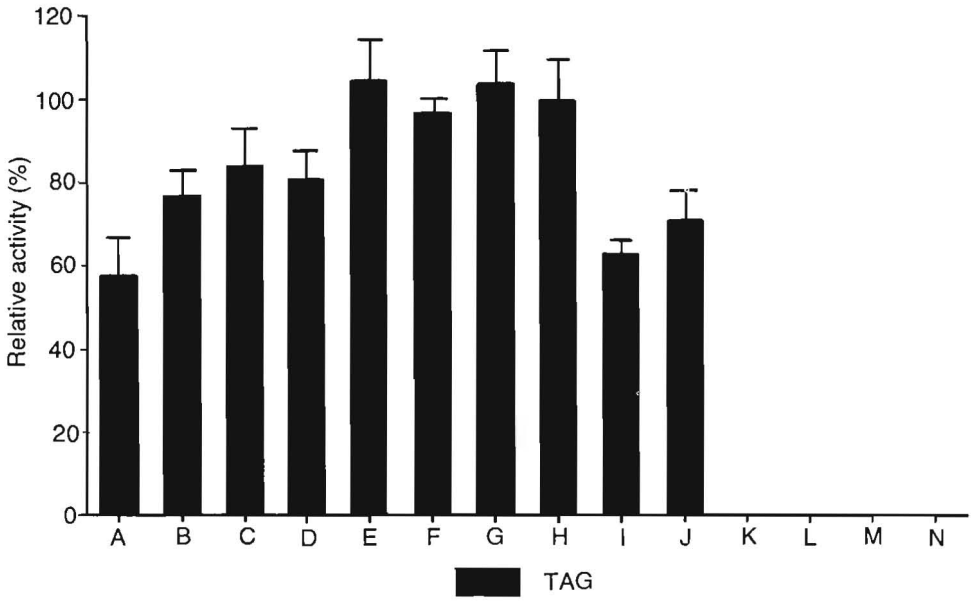

Fig. 7. Bar chart showing linseed lipid body lipase triacylglycerol (TAG) substrate specificity. Activity expressed relative to trilinolein = 100% (10.2 mol FA (mg protein)$^{-1}$ min^{-1}). Data represent an average of 3 replications ± SE.

Linseed lipase is affected little by high concentrations of salts or EDTA (Fig. 8). Pinyon lipase had nearly the same effect with NaCl, KCl, $MgCl_2$ and EDTA (Hammer and Murphy 1993). On the contrary, corn lipid body lipase had reduced activity with Na_3PO_4, $CaCl_2$ and EDTA (Lin *et al.* 1986). Non-ionic detergents reduced linseed lipase, but at low concentrations the effect was not pronounced. On the other hand SDS lowered activity to near zero at low concentration.

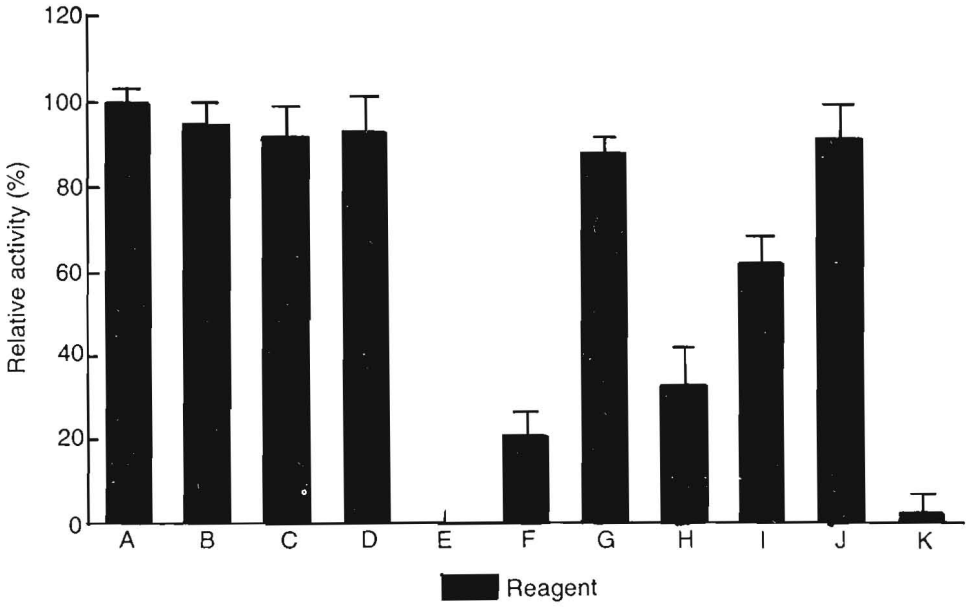

Fig. 8. Bar chart showing the effect of various reagents on linseed body lipase activity. Activity expressed relative to that observed with no additional reagents (100%). Activity measured fluorometrically using ELBP and methylumbelliferly laurate as a substrate. Data represent an average of 3 replications. Bars indicated ± SE.

Conclusion

In linseeds degradation of lipids was accompanied by accumulation of a protein with molecular weight of 42 KDa. This protein accumulated after 36 hours of germination and reached a maximum accumulation after 84 hours of germination, the accumulation of the protein was accompanied with an increase in enzyme activity and a sharp decrease in lipids and linolenic acid contents. The elution of XLBP on Sephacryl S-300 gel permeation column gave an apparent molecular weight of 190 000. This molecular weight was about fourfold the subunit molecular weight of 42 KDa subunit. Antibodies of the subunit protein reacted with XLBP on Western blotting. In addition the immunoprecipitation, using ELBP, indicated that the anti-subunit protein was able to precipitate acid lipase activity from the reaction mixture. The purified protein was reactive against a wide range of triacylglycerols (TAG), specially C18:n sidechain group. These data suggested that the purified proteins was the acid lipase of linseed.

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(Received 26/10/1996;
in revised form 16/09/1997)

مواصفات وتنقية أنزيم الليبيز (ذو الوزن الجزيئي ٤٢ كيلو دالتون) من بذور الكتان النامية

رضا حلمي أحمد سمور

قسم النبات - كلية العلوم - جامعة طنطا - طنطا - مصر

أظهر الفصل الكهربائي لبروتينات الكتان تغير في كمية البروتين ذو الوزن الجزيئي ٤٢ كيلو دالتون كما أظهر أن هذا البروتين يتراكم - بعد ٣٦ ساعة من الانبات - بالتوازي مع الزيادة في نشاط أنزيم الليبيز ، والنقص في كمية الزيوت . ووجد أن البروتين (ذو الوزن الجزيئي ٤٢ كيلو دالتون) هو أحد المكونات البروتينية للأغشية الحاوية للاجسام الزيتية . وقد تم فصل هذا البروتين وتعريفه بإستخدام تقنية الترسيب المناعي كتحت حدة الليبيز . يتفاعل أنزيم الليبيز المستخلص من بذور الكتان النامية مع عدد كبير من التراي أسيل جليسرول ، كما أن نشاطه الامثل عند الرقم الهيدروجيني 4.7 . يتميز هذا الأنزيم بتأثره المحدود بالتركيزات العالية من الاملاح وثنائي أمين الأثيلين رباعي حمض الخليك ، الا أن التركيزات العالية من المنظفات الغير متأينة لها تأثير تثبيطي واضح على نشاط الانزيم . هذه النتائج توضح أن البروتين المعزول (ذو الوزن الجزيئي ٤٢ كيلو دالتون) أنزيم الليبيز الحامضي المسئول عن هدم الزيوت المخزنة في البذور أثناء الانبات .