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Research Article

Purification and Partial Characterisation of an Acid Lipase in Germinating Lipidbody Linseedlings

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Abstract: Electrophoretic analysis of germinating linseed proteins showed a gradual decrease in the quantity of a protein with a molecular weight of 42 kDa. This protein accumulates after 36 h of germination in synchronisation 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 immunoprecipitation as a subunit of lipase. The linseed lipase acted on a wide range of triacylglycerols and had optimal activity at pH 4.7. The activity of the enzyme was slightly affected by a 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.

Key Words: Acid lipase, Triton X-100-solubilised lipid body membrane protein (XLBP), ether-extracted lipid body membrane protein (ELBP).

Introduction

Seeds of some plants store triacylglycerols (TAGs) as small discrete intracellular organelles called oil bodies (Yatsu & Jacks, 1972; Huang, 1985; Stymme & Stobart, 1987; Huang et al., 1991; Siedow, 1991; Tzen et al., 1993; Hammer & Murphy, 1994; Huang, 1996; Millichip et al., 1996; Napier et al., 1996; Fischer & Pleiss, 2003). These oil bodies are used as food reserves for germination and post-germination growth of the seedling.

Lipase, (triacylglycerol acylhydrolase, E.C. 3.1.1.3) is the enzyme catalysing the breakdown of the TAG into glycerol and free fatty acids (Hammer & Murphy, 1994; Shmizu & Nakano, 2003). This enzyme has been purified to homogeneity in only 4 species, the lipid body neutral lipase from the scutella of corn (Lin & Huang, 1984), the glyoxysomal alkaline lipase from castor bean (Maeshima & Beevers, 1985), the major lipase in the megagametophyte of pinyon *(Pinus edulis* Engelm) (Hammer & Murphy, 1993), and the alkaline lipase from the latex of *Euphorbia characios* (Moulin et al., 1994). The corn, castor bean, *Pinus edulis* and *Euphorbia characios* lipases have a protein size of 65, 62, 64, and 38 kDa respectively. It was also reported that the molecular weight of rapeseed lipase was 55 kDa, on the basis of the immunological homology with porcine pancreatic lipase (Beisson et al., 1999).

An analogous lipase, named gastric lipase, is secreted in the stomach of humans and some mammals such as dogs (Roussel et al., 2002). This lipase is stable and active despite the highly acidic stomach environment, and plays an important role in lipid digestion since it promotes the subsequent hydrolytic action of pancreatic lipase in the duodenal lumen. Human gastric lipase is a 50 kDa glycoprotein which is directly secreted as an active enzyme and is the major lipolytic enzyme involved in the digestion of dietary TAG (Miled et al., 2002).

The present study reports on the purification and partial characterisation of the 42 kDa linseed lipase subunit and its relation to TAG degradation.

Materials and Methods

Plant material

Linseeds *(Linum usitatissimum* L., "Giza 5") obtained from the Agricultural Research Center, El-Dokki, Giza, Egypt, were surface sterilised with 70% ethanol for 3 min. After rinsing thoroughly with distilled water, the seeds were transferred to Petri dishes containing 6 ml of distilled water per gram dry weight of the seeds and

germinated at room temperature (23 °C) in the dark (Lin & Huang, 1984). Seeds were harvested every 12 h for 5 consecutive days, during which period the seed coat was removed and a portion was freeze-dried.

Initial localisation study

After removing the seed coats, a portion of the germinated seeds was washed with distilled water, macerated in ice-cold grinding medium (consisting 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 1300x g for 10 min at 5 °C, the supernatant was removed and recentrifuged at 12,000x g for 30 min at 5 °C. Fifty-microlitre 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 & Beevers, 1985).

Enzyme assay

Linseed lipase activity was assayed colorimetrically for the initial localisation, gel permeation, pH optima and TAG substrate specificity studies (Huang, 1985; Hammer & Murphy, 1993). In a Teflon screw-top glass tube, 100 ml of the enzyme fraction and 100 ml of substrate (50 mM trilinolein) suspended in 5% gum acacia by mixing for 30 s with a Tekmar tissuemiser (Tekmar, Cincinnati, OH, USA) were added to 800 ml of assay buffer (100 mM succinate-NaOH, pH 4.7, containing 5 mM DTT) and incubated for 30 min at 25 °C. For pH effects on lipase activity, an assay buffer containing either 100 mM citric acid citrate (pH 2, 3 and 4), tris-malate (pH 5 and 6), or glycine-NaOH (pH 7 and 8) and 5 mM DTT were 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 described by Huang (1985) with a standard curve obtained with linoleic acid. Activity was expressed in nmol fatty acids cleaved min^{-1} mg^{-1} protein. Controls consisted of reaction mixtures with heat-denatured enzyme and controls without substrate.

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

Preparation of lipid body membrane proteins

Germinated seeds (49.95 g) were ground in a Waring blender with 50 ml of 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 centrifuged at 10,000x 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 was almost full and centrifuged again as above (Murphy & Cummins, 1990; Hammer & Murphy, 1993; Edqvist & Farbos, 2002). 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 of detergent-containing buffer medium (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1% Triton X-100, in equal volumes) and orbitally shaken for 3 h at 5 °C. After that the suspension was centrifuged at 50,000x g for 15 min and the centrifuge tubes were carefully placed upright in a freezer at -80 °C. After ca. 16 h, the lipid pad was completely scraped off the frozen supernatant which contained the Triton X-100-solubilised lipid body membrane proteins (XLBPs) (Hammer & Murphy, 1994). For immunoprecipitation study, the lipid pad containing the washed, isolated lipid bodies was resuspended in 20 ml of sucrose-containing buffered medium (20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.2 M sucrose, in equal volumes) and extracted 5 times with a double volume of diethyl ether to remove the triacylglycerols. Diethyl remaining in the final aqueous fraction was evaporated with a stream of N_2. The aqueous fraction was then centrifuged at 100,000x g for 90 min (Lin & Huang, 1984) with the resulting supernatant being the ether-extracted lipid body membrane proteins (ELBPs). XLBP and ELBP had the same distribution of proteins when visualised using SDS-PAGE (Figure 4).

Gel permeation chromatography

Twenty-five millilitres of ELBP (750 mg protein ml^{-1}) was incubated with 1% Triton X-l00 for 1 h at 5 °C, and then concentrated to 4.6 ml using a Centriprep 30 concentrator (Amicon, Danvers, MA, USA). The concentrate was applied to a Sephacryl 5-300 (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 over 6 h (Lin & Huang, 1984). Fractions were assayed colorimetrically for protein and lipase activity.

Electrophoretic purification of 42 kDa protein

The 42 kDa protein from linseed proteins was purified to homogeneity using the protocol used by Hammer & Murphy (1993). In this protocol, 5 ml of XLBP was mixed with 5 ml of SDS-PAGE sample buffer and loaded on two 16 cm x 20 cm x 1.5 mm SDS-polyacrylamide gels (4% stacking, 10% running gels). The gels were run at 25 mA until the bromophenol band was through the stacking gel (2 h) and then at 50 mA for 6 h. The running gels were then rinsed briefly with distilled H_2O and incubated with a nondenaturating $CuCl_2$ stain (ISS Progreen staining system, Enprotech, Hyde Park, MA, USA), and the major 42 kDa protein gel band was excised. The gel slices were electroeluted (Model 422 Electro-eluter, BioRad, Richmond, CA, USA) into 1.4 ml of elution buffer (25 mM Tris 192 mM glycine, 0.1% SDS, in equal volumes) for 5 h at 10 mA $well^{-1}$. The protein was then electrodialysed against the elution buffer without SDS for 0.5 h (at 10 mA $well^{-1}$). The resulting eluate contained the purified 42 kDa lipase subunit.

Protein determination

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

Gel electrophoresis

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

The gel was scanned in a LKB recording laser densitometer equipped with a 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 GLC (Model No. 5730A) GLC.

Antibody preparation

Purified 42 KDa protein was used to immunise rabbits. Purified protein (0.8 mg) was emulsified with Freund's complete adjuvant and injected subcutaneously into each rabbit. This was followed by 5 booster injections with incomplete adjuvant at 15 day intervals.

Immunoglobulin G from serum was purified by the modified method of Hammer and Murphy (1993).

Western blotting technique

Proteins were transferred to nitrocellulose after SDS/PAGE by electroblotting (Towbin et al., 1979). The immobolised proteins on the nitrocellulose sheet were subjected to specific antibodies. After reaction with these antibodies, they were visualised using peroxidase-coupled antibodies and staining with 4-chloro-naphthol carried out using standard methods (Towbin et al., 1979).

Results and Discussion

Experiments with lipid pads showed optimal activity for linseed lipase at acidic pH 4.7, and this was particularly active between 36 h and 84 h of germination (Figure 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 & Murphy (1993) on the megagametophyte of *Pinus edulis.* In contrast, high acid lipase activity was detected in castor bean (*Ricinus communis* L.) dry seeds (Ory et al., 1962; Ory, 1969; Muto & Beevers, 1974). The failure to detect high acid lipase in linseeds may be attributed to the presence of acid lipase inhibitors in the seeds which may mask the activity of acid lipase in vitro. The presence of lipases in dry seeds, of castor bean, *Vernonia galamensis* (Ncube et al., 1995) and rice bran (Bhardwaj et al., 2001) to some extent supports this conclusion and makes the dogma that lipases are absent from dry seeds and are probably synthesised de novo after germination doubtful.

Lipids were extracted from dry and germinating seeds at intervals and their fatty acid compositions were analysed. The data in Figure 2 show that the fatty acids of dry and germinating seeds are palmitic, stearic, oleic, linoleic and linolenic. Linolenic acid represents the major fatty acid of linseed lipids. The quantitative pattern of distribution of fatty acids in linseed lipids is similar to its pattern of distribution in the mature seeds of *Hippophae rhamnoides* L. (Tsydendambaev & Vereschchagin, 2003). The fatty acids follow the same pattern of variation as lipids and their degradation patterns during germination were similar except for those of linolenic acid (Figure 2).

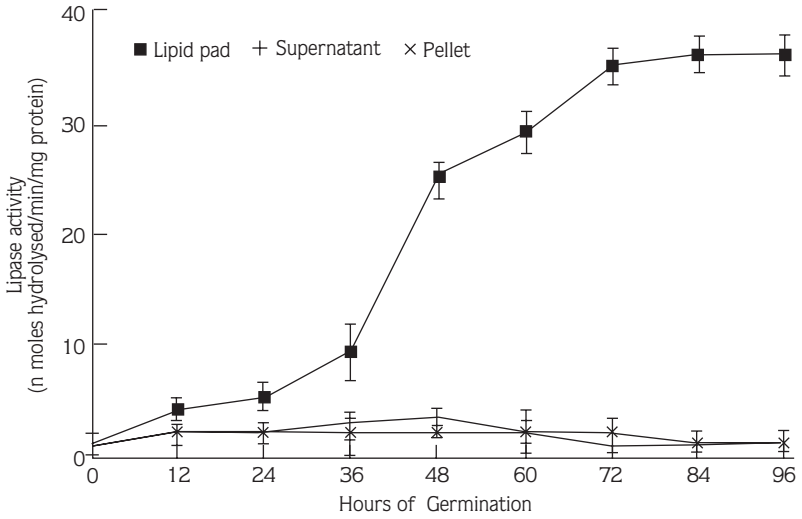

Figure 1. Acid lipase activity of lipid pad, supernatant and pellet from dry (hour 0) and germinating (hours 12-96) linseed. Activity is calculated using the protein concentration of each fraction. Bars indicate ± SE.

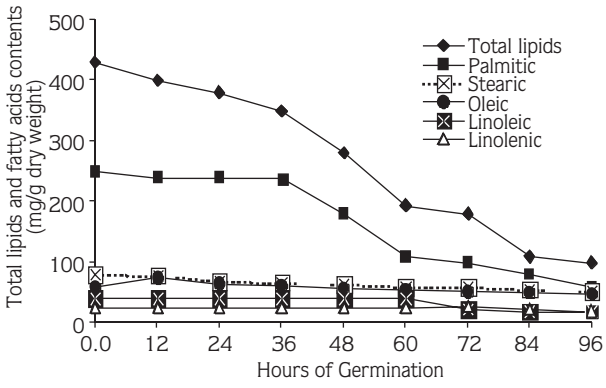

Figure 2. The concentrations of linseed lipids and fatty acids in dry and germinating seeds.

Lipid degradation was accompanied by accumulation of 2 proteins with molecular weights of 65 and 42 kDa (Figure 3a). The likelihood that 65 kDa protein has lipase activity was ruled out because of its presence in the electrophoretic pattern of the extracted meal of the dry seed (Figure 3a), where it was reported that lipase activity is absent before germination and develops during the postgermination stage concomitantly with the disappearance of the storage triacylglycerols (Moulin, 1994; Huang, 1996), as well as its legumin-like protein nature, a fraction of seed storage proteins (Sammour et al., 1994), and its failure to cross react with anti-42 kDa protein, which was able to precipitate acid lipase activity from the reaction mixture (Figures 4, 5). The aforementioned reasons directed our attention towards the 42 kDa protein, the protein which was newly synthesised after germination. Densitometer scans of the tracks in Figure 3b show a 42 kDa protein that accumulated at 36 h and reached a maximum accumulation at 84 h of germination. The accumulation of the 42 kDa subunit at 36 h of germination and its resistance to degradation throughout the course of germination (Figure 3a, b) in combination with 1) the increase in enzyme activity (Figure 1) and 2) the sharp decrease in lipids and linolenic acid (Figure 2) suggest that the 42 kDa protein could be the linseed lipase, and encouraged us to purify this subunit and to study its functional properties.

When XLBPs were separated on a Sephacryl S-300 gel permeation column, they exhibited an apparent molecular weight of 190 kDa. Further purifications using ion exchange chromatography and hydrophobic interaction chromatography were not achieved, as in *Pinus edulis* (Hammer & Murphy, 1993). This failure was apparently due to the fact 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.

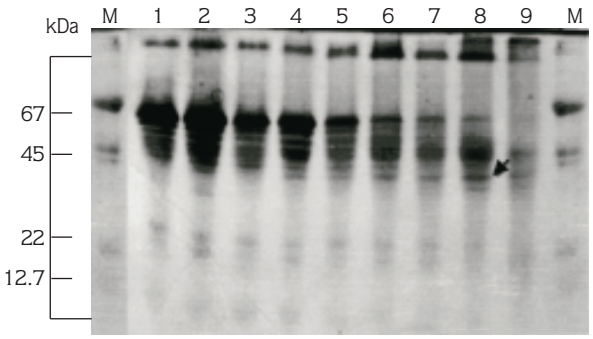

Figure 3a. SDS electrophoretic patterns of germinating linseed. Lane M, mol. wt. markers consisting of BSA (67 KD), ova albumin (45 KD), trypsin inhibitor (22 KD) and cytochrome-C (12.3 KD); lane 1, mature linseed prior to germination; lanes 2-9, after 12 to 96 h.

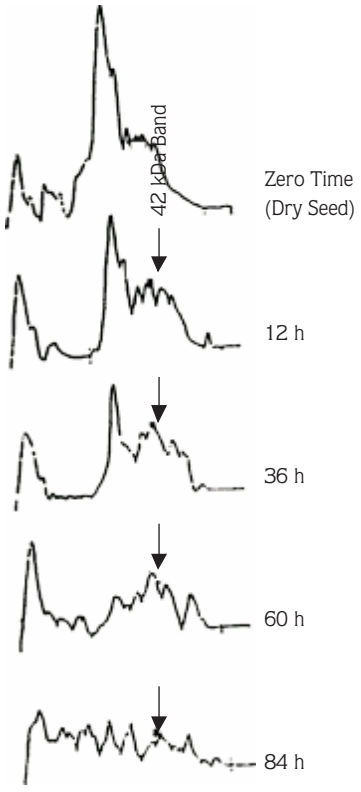

Figure 3b. Scans of gel patterns of germinating linseed. A mature seed prior to germination (dry seed); B, after 12 h; C, after 36 h; D, after 60 h; E, after 84 h.

The molecular weight of linseed lipase was about 4-times the subunit molecular weight. Thus the subunit structure of linseed lipid body acid lipase agrees well with the subunit structure of lipases extracted from other species (Maeshima & Beevers, 1985; Hammer & Murphy, 1993; Beisson et al., 2000). The similarity in subunit structure paralleled the increase in the amount of 42 kDa protein and the rise in lipase activity during germination (Figures 1, 2). For these reasons, the 42 kDa was isolated using preparative SDS-PAGE.

The purified fractions, whose protein components were separated using SDS-PAGE, are shown in Figure 4. Crude cotyledons extract from seed germinated after 84 h (lane 1) was used as a source for the preparation of isolated lipid bodies (Figure 4, lane 2), and the lipid body membrane proteins were solubilised in a 1% Triton X-100 buffer (XLBP, Figure 4, lane 3). The XLBPs were separated using preparative SDS-PAGE, and the 42 kDa protein was isolated by electroblotting from an excised gel slice (Figure 4, lane 4). Antibodies of the 42 kDa protein were highly specific as shown by a Western blot of the SDS-PAGE separated XLBP (Figure 4, lane 5). Immunoprecipitation, using ELBP, indicated that the anti-42 kDa protein was able to precipitate acid lipase activity from the reaction mixture (Figure 5), indicating that the antibody recognised the native lipase enzyme. Therefore, the 42 kDa protein appears to be a subunit of lipase enzyme.

Using ELBP, pH optimum for colorimetric lipase reaction was between pH 4.5 and 4.7 (Figure 6). The pH dependence of colorimetry activity matched that for the lipid body lipase of castor bean (measured by titration), *Pinus edulis* (measured colorimetrically) (Ory, 1969; Hammer & Murphy, 1993), and porcine pancreatic lipase which showed immunological homology with acid lipase in rapeseed (Beisson, 1999).

Linseed lipase was assayed for enzyme activity, using a wide range of triacylglycerols (TAG). The highest activity was on the C18:n side chain group, followed by a slight decrease with C20:0 (Figure 7) and the same trend of activity was reported for papaya (*Carica papaya*) lipase (Gandhi & Mukherjee, 2000). These data also showed that linseed lipase did not hydrolyse mono- or diglycerides. Linseed lipid body lipase was similar in terms of lack of specificity to the lipid body lipases of rapeseed and *Pinus edulis* (Lin et al., 1986; Hills & Murphy, 1988; Hammer & Murphy, 1993).

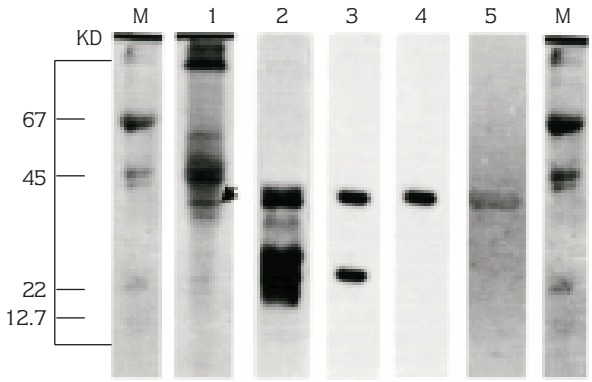

Figure 4. SDS-PAGE (lanes M and 1-4) and Western blot (lane 5) of 84 h germinating linseed. Lane M, marker proteins; lane 1 seed meal extract of 84 h germinating linseed; lane 2, lipid pad; lane 3, XLBP; lane 4. ELBP; lane 5, isolated 42 kDa lipase subunit. The Western blot was probed with rabbit antibodies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

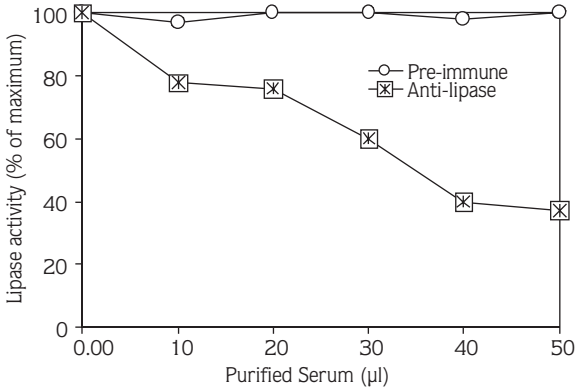

Figure 5. Immunoprecipitation of linseed lipid body lipase by purified anti-42 kDa lipase IgG.

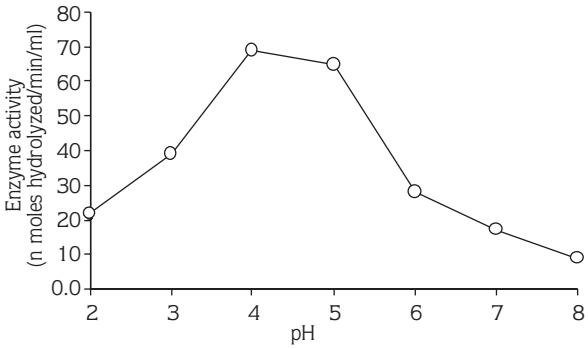

Figure 6. pH effects on 42 kDa protein activity in 100 mM glycine-NaOH buffer containing 5 mM DTT.

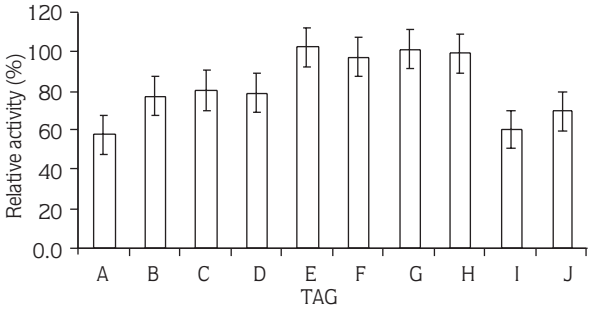

Figure 7. Bar chart showing linseed lipid body lipase triacylglycerol (TAG) substrate specificity. Activity expressed relative to trilinolein = 100% (10.2 mol FA (mg protein)$^{-1}$ min^{-1}). Data represent an average of 3 replications using ELBP ± SE. A, Tricaprin; B Trilaurin; C, Trimyristin; D, Tripalmitin; E, Tristearin; F, Triolein; G, Trilinolein; H, Trilinolenin; I, Triarachidin; J, Tribehenin.

Linseed lipase is little affected by high concentrations of salts or EDTA (Figure 8). Pinyon lipase and rapeseed had nearly the same effect with NaCl, KCl, $MgCl_2$ and EDTA (Lin & Huang, 1983; Hammer & Murphy, 1993; Ben Miled et al., 2000). In contrast, corn lipid body lipase had reduced activity with Na_2PO_4, $CaCl_2$ and EDTA (Lin et al., 1986). Non-ionic detergents reduced linseed lipase, but the effect was not pronounced at low concentrations. On the other hand, SDS lowered activity to near zero at low concentrations.

Conclusion

Linseeds contain acidic lipase with pH 4.7 and a subunit molecular weight of 42 kDa. However, the apparent molecular weight is 190 kDa. This enzyme was detected in dry seed at low concentrations. On germination, it showed a pronounced accumulation after 36 h and reached a maximum after 84 h of germination. The purified enzyme was reactive against a wide range of triacylglycerols (TAGs), especially the C18:n side chain group. Sequencing linseed lipase and determination of its 3-dimensional structure will lead to a better understanding of the structure – function relationships of the enzyme during various hydrolytic and synthetic reactions. This understanding may broaden the use of lipases in industry and medicine and may help in devising efficient methods to overcome the problem of linseed oil instability.

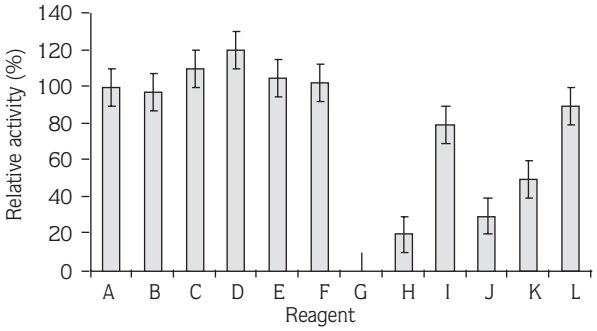

Figure 8. Bar chart showing the effect of various reagents on linseed body lipase activity. Activity is expressed relative to that observed with no additional reagents (100%). Activity measured fluorometrically using ELBP and methylumbelliferyl laurate as a substrate. Data represent an average of 3 replications. Bars indicated ± SE. A, none; B, NaCl (100 mM); C, KCl (100 mM); D, $MgCl_2$ (100 mM); E, $NaHPO_4$ (100 mM)-citric acid, pH5; F, EDTA (10 mM); G, Triton X-100 (0.1%); H, Triton X-100 (0.01%); I, Triton X-100 (0.001%); J, Tween 80 (0.1%); K, Tween 80 (0.01%); L, Tween 80 (0.001%); M, SDS (0.001%).

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