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Characterization of Lupin Seed Lipase

Luis C. Sanz & Jose M. Olias*

UEI Fisiologia y Tecnologia Post-recolección, Instituto de la Grasa y sus Derivados, CSIC, Aptdo 1078, 41012-Sevilla, Spain

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ABSTRACT

Crude lipase has been extracted from ungerminated lupin seed. The lipase has an optimal activity at pH 5·0, an optimum temperature of 25°C and an activation energy of 1·32 kJ mol^{-1}, showing an induction period of 10 min. A crude extract lost more than 70% of lipase activity after 24 h. Lipase activity is stimulated by the concurrent presence of potassium (10 mM) with calcium (1 mM) or magnesium (1 mM) ions. The enzyme exhibits a certain specificity in releasing fatty acids from the primary rather than from the secondary position of triglycerides of lupin oil. At the primary position it is more active on saturated than on unsaturated fatty acids.

INTRODUCTION

The increasing demand for oilseed proteins for human consumption and animal feed has stimulated the search for new sources. Lupin is being investigated in several countries as a potential producer because of its relatively high protein (35–40%) and oil (8–12%) contents, high productivity and low cost (Hill, 1977; Watkin, 1979; Zevallos, 1980; Huesa *et al.*, 1985*a*). A characteristic of lupin triglycerides is a high unsaturated fatty acid content (74–82%) of which 26–45% is polyunsaturated (Cerletti & Duranti, 1979; Huesa *et al.*, 1985*b*). This high level of unsaturated fatty acids renders lupin oil susceptible to oxidative rancidity and creates a storage problem if high quality in the final products is to be retained. The degradative process has been studied in soybean (Rackis *et al.*, 1979), and the formation of free fatty

* To whom all correspondence should be addressed.

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acids appears to be closely associated with the process of lipid oxidation (Singleton & Pattee, 1980). The action of lipase is implicated in the release of fatty acids, as the conditions of normal storage and handling are not conducive to non-enzymatic hydrolysis.

Most investigations on plant lipases have been carried out on oleaginous seeds in which lipase activity is generally found to become prominent upon germination (Huang & Moreau, 1978; Rosnitchek & Theimer, 1980; Lin *et al.*, 1982, 1986; Lin & Huang, 1983; Hassanien & Mukherjee, 1986). Published works, directly concerning lipase activity in ungerminated seeds, are scarce (Ory *et al.*, 1960; Dundas *et al.*, 1978; Kermasha *et al.*, 1986), although there are some publications concerning changes in the fatty acid composition of the lipids of oilseeds with time (Singleton & Pattee, 1980; Yao *et al.*, 1983). Considering the possible relation between lipase activity and the development of off-flavour in oilseeds during storage via the autoxidation of the fatty acids released, a study was undertaken to assess the lipase activity of lupin seed. The purpose of this work was to devise a methodology for the preparation of an active lipase extract from lupin seed, to characterise the enzyme activity as a function of pH, to study the effects of ions and temperature, and to determine the substrate specificity.

MATERIALS AND METHODS

Seeds of *Lupinus albus* cv *multolupa* were used in the present study. The seeds were harvested in 1987 and subsequently stored at room temperature. Ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), triethanolamine, linoleic acid, tristearin and porcine pancreatic lipase were obtained from Sigma Chemical Co., St Louis, MO, USA, 1,5-diphenylcarbazide and cupric nitrate were from Fluka Chemie AG, Buchs, Switzerland. Other routine chemicals and solvents of analytical grade were products of Merck, Darmstadt, FRG.

Preparation of enzyme extracts

The seeds were soaked in water overnight at room temperature. The cotyledons were homogenised in a Sorvall omnimixer, in batches of 10 g wet weight, in two volumes of grinding buffer, 50 mM sodium phosphate, pH 6·8, containing 0·2 mM EDTA and 0·3 mM DTT. Grinding was done in five 20 s periods. The homogenate was filtered through four layers of gauze and centrifuged for 30 min at 30 000*g* at 4°C. Protein was measured by the Bradford method (Bradford, 1976) with crystalline ovalbumin as standard.

Enzyme assay

Lipase activity was measured by a colorimetric method. The fatty acids released were converted to copper soaps and quantified using 1,5-diphenylcarbazide as the colour reagent (Nixon & Chan, 1979). The 1·0 ml reaction mixture contained 0·1 M acetate buffer, pH 5·0, or other buffer as stated, 5 mM DTT, 2·5 mM substrate and 0·1 ml enzyme preparation (50 mg protein/ml). The substrate, lupin triglycerides purified from lupin oil, was emulsified in 5% gum arabic for 1 min at high speed with a sonicator. The emulsions were prepared immediately before use. The reaction was carried out at 30°C in a shaker water bath, stopped at 40 min by adjusting the pH to 3 with 4N HCl, and the fatty acid content in aliquots of the reaction mixture was measured. A standard curve for linoleic acid was constructed. The unit of lipase activity is defined as the amount of enzyme required to liberate 1 μmol of fatty acid from the triglyceride emulsion under the above conditions of assay.

Lipid analysis

The products of lipolysis were extracted and fractioned by TLC on silica gel G. The plate was developed with hexane:diethyl ether:acetic acid (70:30:1, v/v/v), and the band of fatty acids was scraped off, eluted with diethyl ether and esterified with diazomethane. The quantitative analysis of the individual fatty acids was carried out by GLC on a column 2 m long and $\frac{1}{8}$ i.d. packed with 15% DEGS on Supelcoport 80–100 mesh. The oven temperature was 180°C. Methyl heptadecanoate was used as the internal standard. For determining intraglyceride distribution of acyl groups in lupin triglycerides, fatty acids from lipolysis products by pancreatic lipase were analysed in monoglyceride fractions.

RESULTS AND DISCUSSION

A common feature among lipases from diverse seeds is that the enzyme activities are absent in ungerminated seeds and increase during post-germination (Huang, 1984). Initial work was directed at determining the ability of crude extracts from ungerminated lupin cotyledons to release free fatty acids from lupin triglycerides. The homogenate obtained as stated in the 'Methods' section was centrifuged yielding a fat layer (lipid bodies), a supernatant liquid and a pellet. The fat layer was carefully removed with a spatula and the supernatant separated from the pellet. The fat and the pellet were discarded and the supernatant was saved. Aliquots of the enzyme

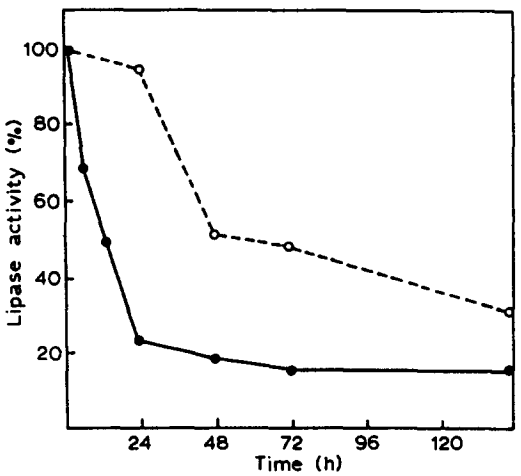

Fig. 1. Effect of storage at 4°C on the activity of lipase of lupin crude extract (●) and filtered crude extract by Sephadex G-25 column (○).

preparation were used immediately after their isolation for the assay of lipase activity. The supernatant (crude extract) exhibited an acid lipase activity.

To study the stability of the lipase preparation, the crude extract was kept at 4°C and assayed over a 6-day period. The loss of activity was noticeable within 6–8 h and more than 70% of activity was lost at 24 h. Apparently, the enzyme was inactivated soon after tissue homogenisation or its activity was somehow inhibited. Naturally-occurring inhibitors of lipases are relatively unknown. Wang and Huang (1984) have found inhibitory factors of lipase activities in extracts of cotyledons of soybean, sunflower, cucumber and peanut seeds. They suggest that the inhibitors are proteins which bind to the surface of the substrate micelles. When crude lipase extract from lupin was purified by passage through a Sephadex G-25 column (2·5 × 30 cm, 40 ml min^{-1} grinding buffer), the stability was extended. The results are summarised in Fig. 1. Thus, if the inhibitors are proteins, their apparent molecular weights should be 5000 or smaller.

Characterization and properties

The fractions eluted in the void volume of the Sephadex G-25 column were pooled and used for subsequent enzyme characterisation. The effect of pH on the relative rate of hydrolysis of lupin triglycerides was studied using acetate (range 4–6), phosphate (range 6–8) and borate (range 8–10) buffers (0·1 M). The enzyme exhibited a narrow range of pH activity with a maximum at pH 5·0. The enzyme had no activity below pH 4·0 or above pH 7·0. Both acid and alkaline lipases have been found in seeds. Huang and Moreau

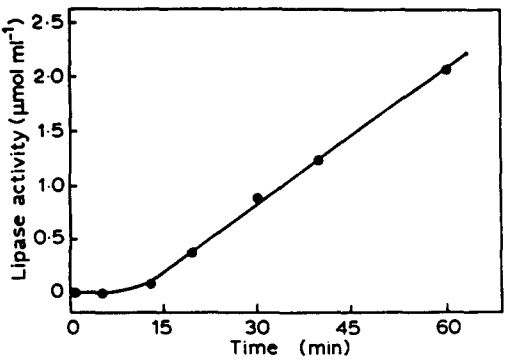

Fig. 2. Effect of reaction time on the extent of hydrolysis of lupin triacylglycerols by lupin lipase.

(1978) have studied lipolytic activity in several germinating oilseeds. With the exception of castor bean, none contained acid lipase activity, but all had an alkaline lipase activity in their storage tissues. The acid lipase in castor bean is associated with the spherosome membrane and is present in the dry seed. Lupin lipase, in this work an acid one, is present in dry seed but its subcellular location is different inasmuch as lipid bodies were discarded.

The effect of time of reaction on the rate of hydrolysis of lupin triglycerides by the lipase preparation is shown in Fig. 2.

After an induction period of 10 min, the hydrolysis proceeds at a linear rate up to about 60 min. This period might be due to the coupling of the enzyme in the interface substrate-water.

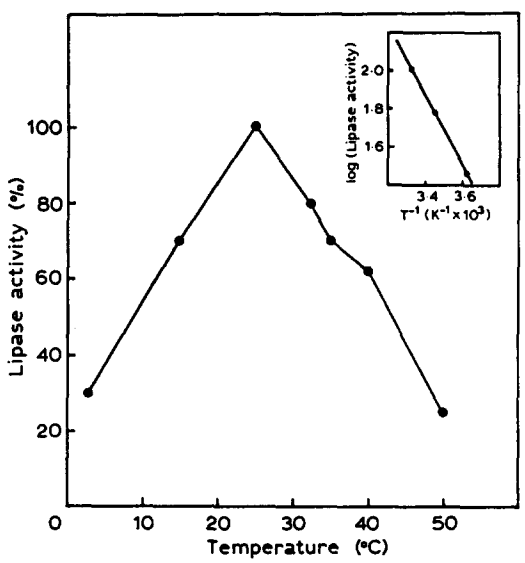

Fig. 3. Effect of temperature on lupin lipase activity. Inset, activation energy determination according to Arrhenius.

The influence of temperature on the enzyme activity was determined following incubation for 60 min. The optimal temperature was 25°C. The thermal activation constant was calculated for incubation temperatures between 0 and 25°C and was found to be 1·32 kJ mol^{-1} (Fig. 3).

Monovalent ions suppress enzyme inhibition by interfacial charge effects (Benzonana & Desneulle, 1968). On the other hand, divalent cations enhance lipase activity acting as fatty acid acceptors, since free fatty acids usually inhibit lipase (Brockerhoff & Jensen, 1974). Studies of the effect of metal ions on lipase activity have been made individually. Inasmuch as monovalent and divalent ion effects seem to be complementary, a study of potassium–calcium and potassium–magnesium was carried out. Chloride salts were added to the lipase extract and incubated for 6 h before the enzyme assay. The most powerful effect on the activity of lupin lipase was obtained with mixtures of potassium–calcium. The presence, in the lipase extract, of 10 mM KCl and 1 mM $CaCl_2$ enhanced the relative activity by 50% and 10 mM KCl with 1 mM $MgCl_2$ by 40%.

Positional specificity

To assess the positional specificity of lupin lipase, 25 mg of lupin triglyceride was emulsified with 5% gum arabic in 10 ml of 0·1 M acetate buffer, pH 5·0, and mixed with 10 ml of crude extract. The reaction mixture was incubated at 30°C in a shaker water bath for a period of 40 min. The products of hydrolysis were examined by TLC. The lipolysis products obtained were exclusively composed of monoacylglycerols and diacylglycerols and fatty acids.

The substrate specificity of a lipase is defined by its positional specificity, its stereospecificity or its preference for longer or shorter-chain saturated or unsaturated acids. To determine the specificity of lupin lipase, fatty acids of

TABLE 1
Percentage[a] of Each Fatty Acid Relative to the Total Fatty Acids in Lupin Triglyceride

Acid	*Triglyceride*[b]	*Primary position*	*Secondary position*	*Fatty acids from hydrolysis by lupin lipase*
C16	8·6	12·5	0·7	25·5
C18	2·6	3·8	0·2	9·7
C18:1	53·7	49·6	61·9	36·6
C18:2	23·1	20·2	29·0	16·5
C18:3	11·7	13·7	7·9	11·4

[a] Average of three determinations.
[b] Fatty acid percentage by transesterification with CH_3ONa and HCl—CH_3OH.

lipolysis were quantified by gas chromatography after esterification with diazomethane. The positional distribution of fatty acids in triglycerides of lupin oil, used as a substrate, was determined by porcine pancreatic lipase. The results of this analysis are shown in Table 1.

As judged from the pattern of hydrolysis of lupin triglycerides, lupin lipase exerts some degree of preference in releasing fatty acids from the primary rather than from the secondary position of a triacylglycerol. At the primary position, the enzyme preferentially releases more saturated than unsaturated acids. To check this preference, lipase activity was estimated using tristearin and lupin triglycerides as substrate. Lipase was slightly more active towards tristearin (120%) than towards triacylglycerols of lupin oil (100%). The results of relative activities on the saturated and unsaturated triglycerides (lupin oil has more than 80% of unsaturated fatty acids) seem to support the earlier suggestion that lupin lipase is slightly more active towards saturated fatty acids.

In conclusion, it has been shown that the activity of lipase in lupin seed is adequate for the release of fatty acids and changing the profile of its lipids. The resulting free fatty acids may be the main components undergoing autoxidation to produce acids, ketones, aldehydes or other substances during storage or processing. These degradative products can react with amino acids impairing flavour and/or lowering the nutritive value of the lupin seed itself or the food into which it is incorporated.

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