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Acetone Powder From Dormant Seeds of *Ricinus communis* L

Lipase Activity and Presence of Toxic and Allergenic Compounds

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Abstract

The influence of several factors on the hydrolytic activity of lipase, present in the acetone powder from dormant castor seeds (*Ricinus communis*) was evaluated. The enzyme showed a marked specificity for short-chain substrates. The best reaction conditions were an acid medium, Triton X-100 as the emulsifying agent and a temperature of 30°C. The lipase activity of the acetone powder of different castor oil genotypes showed great variability and storage stability of up to 90%. The toxicology analysis of the acetone powder from genotype Nordestina BRS 149 showed a higher ricin (toxic component) content, a lower 2S albumin (allergenic compound) content, and similar allergenic potential compared with untreated seeds.

Index Entries: 2S albumin; castor seeds; lipase; ricin; *Ricinus communis;* acetone powder.

Introduction

Lipases (glycerol ester hydrolase, enzyme comission [EC] 3.1.1.3) are defined as a group of enzymes that are capable of catalyzing the hydrolysis of long-chain triacylglycerols into glycerol and free fatty acids, although they can also utilize other substrates such as medium or short fatty acid esters. Because of the hydrophobic nature of their substrates, lipases generally act in an aqueous–organic interface. Apart from their hydrolytic activity, lipases may also present reverse activity (esterification and transesterification reactions) in water-restricted environments, such as organic solvents. Their capacity to carry out all these transformations with

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a high chimio-, regio-, and enantio-specificity means that lipases have great potential as biocatalysts in technological applications, such as in detergents, foods, pharmaceuticals, oleochemicals, and other industries *(1,2)*.

Lipases are generally found in animals, plants, and microorganisms, but microbial enzymes have been studied the most and applied most to industrial processes. However, in recent years plant lipases have also attracted the attention of researchers. There still exists a huge diversity of plants to be explored and the discovery of lipases with different specificities and stability would extend these enzymes' range of application. Plant lipases generally exhibit a particular specificity, usually a substrate specificity (more pronounced than in microbial enzymes), and are a low-cost raw material. Most of the literature on plant lipases concerns oil seed lipases, of which the most studied seeds are probably from oats, colza, and castor seeds. Lipases from the dormant seed of the castor plant (*Ricinus communis*) could be an option of some interest for industrial applications, given that they are found in large quantities in the ungerminated seed, whereas lipases from oil seeds generally need the seed to start germinating for them to be synthesized, and the quantities produced are very small *(3,4)*. Additionally, the castor bean grows throughout Brazil, it is easy to cultivate and is attracting interest because of its oil, which presents a challenge in finding the best ways to exploit the seeds using the latest scientific and technological approaches.

The lipase activity of dormant castor seeds has been recognized for over a century. Lipases are found in association with the lipid body (small intracellular organelles that store the oil) membranes, and are probably anchored by their hydrophobic region close to the N-terminal end *(4,5)*. The lipase accounts for 5% of the total proteins in the lipid bodies and its molecular weight has been estimated at around 60 kDa *(6)*. Recently, Eastmond *(4)* cloned the lipase of the dormant castor seed with the purpose of clarifying the role this enzyme plays in starting germination; however, its physiological function is still unclear and a new hypothesis is that it might be involved in defending the seed against parasites.

The presence of compounds with a high-toxicity (ricin) and pronounced allergenicity (2S albumin isoforms) *(7,8)* in castor seeds should be taken into account whenever industrial uses are planned for castor seed derivatives, including lipid biotransformation. Ricin is the most lethal of the toxins present in castor cake; it is a member of the family of ribosome-inactivating proteins constituted of two polypeptide chains: the A-chain hydrolyses a conserved region of the 28S rRNA, blocking protein synthesis and bringing about cell death, whereas the B-chain exhibits lectin properties by which the toxin is endocyted *(8,9)*. The castor seed allergen is a non-toxic and unusually stable protein belonging to the 2S albumin class, which exhibits an exceptional capacity to enhance individuals' sensitivity to small concentrations of dust from castor seeds or castor cake *(7)*. The aim of this work was to characterize the lipase activity present in dormant castor seed

acetone powder and to evaluate the acetone treatment for inactivating toxic and allergenic compounds. The castor oil genotypes with the greatest potential as sources of lipases were also selected.

Materials and Methods

Castor Seeds

The castor bean seeds were supplied by Embrapa Cotton Research Center in Campina Grande, Brazil, and stored at 4°C until use.

Extraction of the Acetone Powder

One hundred and fifty milliliter acetone at 4°C was added to 10.0 g seeds and the mixture was blended (Turmix blender; ARNO) and sieved to obtain particles smaller than 500 μm. These were washed with 300 mL acetone at 4°C and incubated with 150 mL acetone for 16 h. After incubation they were washed with 150 mL acetone. The fat-free powder was left in an open flask for 24 h to complete the removal of the residual acetone and then stored at 4°C until use.

Lipase Assay

Lipase activity was determined by adding 90.0 mg acetone powder to 10 mL emulsion consisting of tributyrin* (5% [w/v]), Triton X-100* (25% [w/v]), and acetate buffer, 0.05 *M*, pH 4.0 (50% [v/v]). This was incubated at 37°C* for 3, 5, and 7 min under agitation (200 rpm). The reactions were stopped by adding 20 mL ethanol and the fatty acids were extracted under agitation (200 rpm) for 10 min and titrated until the end point (pH 11.0) with a NaOH solution (0.04 *N*). The blank assays were performed by adding the acetone powder after the ethanol had been added. The activity (U) was calculated from the α of the graph (μmol free fatty acids vs reaction time), which resulted in the specific activity (U/g acetone powder). One lipase activity unit (U) was defined as the amount of enzyme that produced 1 μmol fatty acid per min under the assay conditions. The reactions were performed in triplicate.

Effect of Different Factors on Lipase Activity

The assays were carried out as previously described except when evaluating the influence of pH.

- *Temperature:* 25, 30, 35, 40, 45, and 50°C.
- *Type of emulsifier:* Triton X-100 (25% [w/v]) and gum arabic (5% [w/v]); the reaction times were 10, 20, 30, and 40 min.
- *Type of substrate:* olive oil, sunflower oil, castor oil (the oils were from local stores), trycaprylin and tributyrin (purchased from Sigma); reaction emulsions contained 10% (w/v) substrate.

*These conditions were changed in some assays.

- *Comparison among different genotypes:* acetone powder from castor genotypes Nordestina BRS 149, Brejeira CNPAM 93-168, CSRN 393, Pernambucana SM-5, and CSRD-2.
- Storage stability: acetone powder was stored for 7 mo at 4∞C before use.

Two experiments were performed to evaluate the influence of pH.

- Hydrolysis catalyzed by the acetone powder: ten milliliter sunflower oil was incubated with 50.0 mg acetone powder in 10-mL buffer (acetate pH 4.0 or phosphate pH 7.2) at 50∞C for 68 h. The blank assays were performed by incubating the oil and buffer solution only.
- *Autohydrolysis:* the action of the endogenous lipase on the seed oil was verified by incubating 1 g ground seed with 10-mL buffer (acetate pH 4.0 or phosphate pH 7.2) at 50°C for 1 h under agitation. The control or blank assay was performed with ground seeds and no addition of a buffer. The thin-layer chromatography (TLC) of the sunflower oil reactions (eluted with 70/30/1 hexane/diethyl ether/acetic acid) and castor seed oil reactions (eluted with 40/60/1 hexane/diethyl ether/acetic acid) were visualized by spraying the TLC plate with a (1 : 1) saturated solution of cupric sulphate : phosphoric acid 85%, followed by 5 min at 180°C.

Determination of Toxic and Allergenic Compounds

In these experiments untreated Nordestina BRS 149 seed and acetone powder were compared.

Determination of 2S Albumin Content

3.2 mL water at 80°C was added to 16.0 mg acetone powder or ground castor seed and was incubated at 60°C for 6 h under agitation. After decantation, 500 µL from the upper layer was removed and submitted to gel-filtration chromatography using a 50 × 1.5 cm^2 Sephadex G-50 column, eluted with trifluoroacetic acid (TFA) 0.1%, at 0.7 mL/min. 200 µL was taken from the fraction containing 2S albumin and injected into the C18 reverse phase chromatography column. The sample was eluted using a solvent gradient consisting of solvent A (TFA 0.1%) and solvent B (acetonitrile 80% and TFA 0.1%): 0–10 min 0% of B; 10–40 min 0 to 80% of B; 40–45 min 80% of B; 50–55 min 80 to 0% of B.

Immune-Detection of 2S Albumin

The fractions containing 2S albumin that originated from the reverse phase chromatography were concentrated 15-fold and 10 µL of this concentrate was spotted on a nitrocellulose membrane using 2% milk powder as a blocker. After exhaustive washing, an anti-2S albumin rabbit antibody (dilution 1 : 500) and a secondary antibody were added. The primary antibody was not added to the negative control so that any unspecific reactions could be observed.

Evaluation of Allergenic Properties

The mast cells used came from Wistar rats, which were put down by CO_2 asphyxia. The peritoneum cavity was washed with 20 mL Dulbecco's Modified Eagle Medium containing heparin, and 15 mL was incubated in a Petri dish at 37°C for 30 min, after which time 2/3 of the upper phase was discarded. The mast cells (100 µL) resulting from sedimentation in the Petri dish were treated with:

1. Only the preimmune rat serum.
2. Only the anti-2S albumin serum.
3. Anti-2S albumin serum and 2S albumins (10 µg/mL).
4. Negative control (without any treatment).

The pool of IgE obtained from immunized RA/thor rats was diluted at 1 : 100 in the mast cell suspension and subsequently incubated at 37°C for 1 h. In order to observe degranulation, 10 µL cell suspension was incubated for 15 min with 10 µL of a solution containing 0.1% toluidine blue, 10% formaldehyde, and 1% acetic acid at pH 2.8. The count of whole and damaged cells was performed with a microscope using a Neubauer (BOECO, Germany) chamber.

Determination of Ricin Content

Sixteen milligrams acetone powder or ground castor seed was dissolved in 3.2 mL deionizer and distilled water and incubated at 80°C for 4 h under stirring and a further 16 h at room temperature. The proteins were analyzed in 12% (w/v) SDS-polyacrylamide gel (SDS-PAGE) according to the methodology described by Laemmli *(10)*. Sample preparation: 40 µL sample buffer containing SDS (10%) and β-mercaptoethanol (5%) was added to 80 µL protein extract, and 35 µL of this mixture was used to run the SDS-PAGE.

Results and Discussion

Effect of Different Factors on Lipase Activity

Acetone powder of genotype Nordestina BRS 149 was used to study the influence of pH, emulsifier type, substrate type, and temperature on the lipase activity of dormant castor seeds.

Influence of pH

Figure 1A shows the TLC of hydrolysis conducted in acid and neutral reaction media by the acetone powder on sunflower oil. The results for the reaction at pH 4.0 (lane b) show the hydrolysis products: fatty acids (retention factor [Rf] = 0.76) and partial triacylglycerols (Rf 0.60, Rf 0.50, and Rf 0.14). At pH 7.2 (lane c), no reaction took place so the pattern was much the same as for the starting oil (lane a). This result is in agreement with Eastmond *(4)* who only found significant activity from cloned dormant castor seed lipase at pH 3.5–5.0. The same pH influence was observed for autohydrolysis (Fig. 1B). The reaction at pH 4.0 (lane c) shows fatty acids

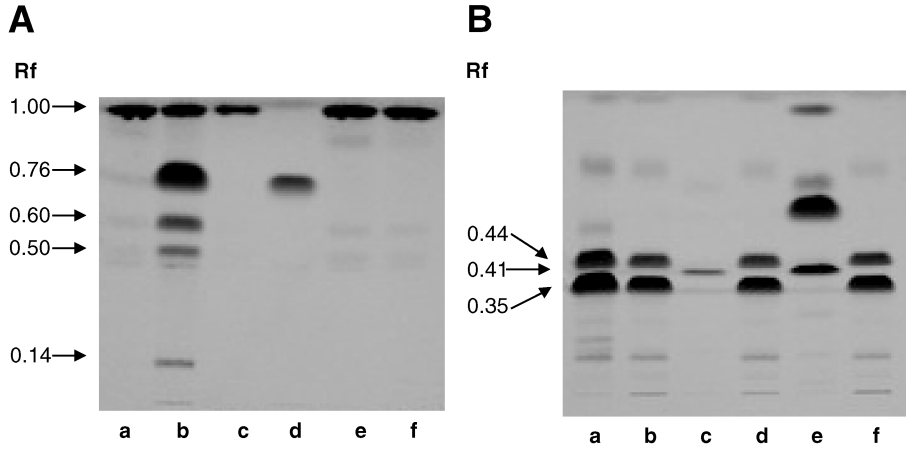

Fig. 1. (A) TLC of sunflower oil hydrolysis by acetone powder in neutral and acid media: (a) sunflower oil, (b) hydrolysis at pH 4.0, (c) hydrolysis at pH 7.2, (d) standard fatty acid, (e) negative control at pH 4.0, and (f) negative control at pH 7.2. **(B)** TCL of autohydrolysis in neutral and acid media: (a) commercial castor oil, (b) extracted castor seed oil, (c) hydrolysis at pH 4.0, (d) hydrolysis at pH 7.2, (e) reference sample of ricinoleic acid (Rf 0.41), and (f) negative control of ground seed hydrolysis.

(Rf 0.41), but the reaction at pH 7.2 (lane d) only shows the seed oil triacylglycerols (Rf 0.44 and Rf 0.35), as does the starting oil (lane b).

Influence of Emulsifier Type

Considerable lipase activity was observed of the acetone powder on tributyrin (5% [w/v]) emulsified with gum arabic (5% [w/v]) or Triton X-100 (25% [w/v]): 139 ± 6 U/g and 220 ± 7 U/g, respectively. The almost twice as high-activity obtained with Triton X-100 may have been caused by alterations to the substrate aggregation state and/or changes to the enzyme's structure *(11)*. One hypothesis is that the lipase's capacity to anchor to the interface may be greater because of the lower interfacial tension of emulsions prepared with Triton X-100 *(12)*, or this lower tension may cause a larger interfacial area that makes a greater amount of lipid available for lipase activity. Some enzymes, not just lipases, have been reported to be activated by triton-X 100 *(13–15)*, possibly by molecular structural changes to a more compact protein *(14)*. The result could also be associated to the composition of the emulsifiers. For example, higher lipase activity has been reported in the presence of nonionic emulsifiers (like Triton X-100) than with ionic emulsifiers (like gum arabic) *(15)*, and castor seed lipase seems to be partially inhibited by some ions *(16)* present in gum arabic (the gum is a plant exudate and has no fixed composition) *(17)*.

Influence of Substrate Type

The lipase activity of acetone powder with different substrates (10% [w/v] substrate emulsified with 25% [w/v] Triton X-100) was: olive oil

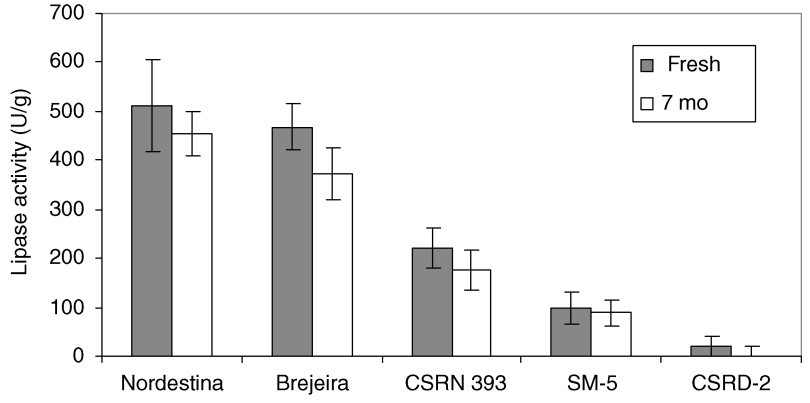

Fig. 2. Lipase activity of the fresh acetone powder from different castor genotypes and after storage for 7 mo at 4°C. Reactions were conducted using tributyrin (5% [w/v]) emulsified with Triton X-100 (25% [w/v]) in a buffer, pH 4.0, at 37°C, catalyzed by acetone powder from the different castor genotypes.

28 ± 3 U/g, sunflower oil 24 ± 2 U/g, castor oil 28 ± 2 U/g, tricaprylin 56 ± 7 U/g, and tributyrin 811 ± 54 U/g. The lipase exhibited a marked specificity for triacylglycerols formed by shorter chain fatty acids such as tributyrin (C4). As reported for *Geotrichum candidum* lipase, the chimio-specificity could be attributed to a few aminoacid residues both at the entrance and at the bottom of the active site cavity *(18)*. However, some authors believe that greater lipase activity on a smaller substrate may be caused by the increased water solubility of these short chain compounds, which would create a larger interfacial area and a less pronounced inhibition by products because the free fatty acids would have a stronger tendency to go to the aqueous phase than those of medium and long chains *(19)*.

Influence of Temperature

The lipase activity of the acetone powder (5% [w/v] tributyrin with 25% [w/v] Triton X-100) was higher in the 25 (429 ± 9 U/g) to 35°C (368 ± 6 U/g) range, and the optimal temperature was 30°C (429 ± 9 U/g). The higher temperatures resulted in lower activities: 216 ± 5 U/g at 40°C; 57 ± 4 U/g at 45°C; and not detectable activity at 50°C.

Lipase Activity of Different Castor Genotypes and Storage Stability

The lipase activity presented by the acetone powders from the different castor genotypes (Fig. 2) shows a great variation (96%) among them. This pronounced variation in lipase activity among different genotypes has also been observed for other plants, such as *Carica papaya* latex (92%) *(20)* and palm fruit (70%) *(21)*. The most promising castor genotypes as sources of dormant seed lipases were Nordestina BRS 149 (511 ± 63 U/g) and Brejeira CNPAM 93-168 (468 ± 47 U/g) (Fig. 2). After the acetone powders had been stored for 7 mo at 4°C, their remaining lipase activity (Fig. 2) was

Table 1
Ricin Content, 2S Albumin Content, and Allergenic Properties of Acetone Powder and Untreated Castor Seed of Genotype Nordestina BRS 149

Material	Ricin (%)[a]	Albumins 2S (%)[a]	Mast cells degranulation (%)[b]
Castor seed	0.85	2.1	43
Acetone powder	2.15	0.7	45

[a]gram per 10.0 g of material.
[b]number of mast cells degranulated per 100 mast cells.

80–90% of the fresh powders, except for genotype CSRD-2, which lost all its activity *(22)*.

Toxic and Allergenic Compounds in Acetone Powder

The ricin protein was isolated by water extraction and gel-filtration chromatography then detected by SDS-PAGE (Table 1); 0.85% (gram per 10.0 g of material) content was found in whole seeds and 2.15% in acetone powder. The higher toxin concentration found in acetone powder may be owing to the elimination of the oil from the seed (about 50% of the seed mass). However, investigations into acetone powder toxicity are recommended because the ricin molecule could be inactive yet present. The 2S albumin was isolated by gel-filtration and reverse phase chromatography (Table 1) and its content dropped by 2.1 to 0.7%. This reduction was confirmed by immune-blot analysis (data not shown). Castor bean allergens demonstrate extreme stability and there are few methodologies for removing the allergen from the cake. Recently, Kim *(23)* proposed a drastic heat- and NaOH- or NaOCl-based treatment to reduce antigenic activity, so the acetone treatment proposed here could be another option. The biological assays to evaluate the allergenic properties of acetone powder based on mast cell degranulation presented very similar degranulation percents to untreated castor seeds (approx 43%) (Table 1).

Conclusions

The acetone powder from dormant seeds of *R. communis* contains a lipase that is only active in an acid pH. This enzyme activity is stimulated by the Triton X-100 emulsifier, and has a marked specificity for short-chain substrates and a pronounced variability among castor genotypes. The acetone powder also has good storage stability. The ricin content was higher in acetone powder. However, a toxicity investigation is needed to evaluate whether the protein is still in its active form. Given that 2S albumins are stable proteins, and that inactivation treatments have not yet been described, a reduction of their level by acetone treatment could be a promising technique for eliminating these allergenic proteins.

Acknowledgments

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