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ORIGINAL PAPER

Effect of Surfactants and Polyethylene Glycol on the Activity and Stability of a Lipase from Oilseeds of *Pachira aquatica*

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Abstract Lipases from oilseeds have a great potential for commercial exploration as industrial enzymes. Lipases are used mixed with surfactants in cleaning and other formulated products, and accordingly, both components must be compatible with each other. This work presents the results of the effects of anionic, cationic and nonionic surfactants, polyethylene glycol and urea on the activity and stability of a lipase extracted of oilseeds from *Pachira aquatica*. The enzyme was purified and the spectrophotometric assays were done using *p*-nitrophenyl acetate (*p*-NPA) as substrate pH 7.5 and 25 °C. The activity was significantly enhanced by the cationic surfactant CTAB. Bile salts increased the lipase activity in the tested concentration range, whereas anionic and nonionic surfactants showed an inhibitory effect. Aqueous solutions of PEG activated the lipase and maximum activation (161%) occurred in PEG 12,000. This effect on lipase that can be due to exposition of some hydrophobic residues located in the vicinity of the active site or aggregation.

Keywords Oilseeds · Lipase · Surfactants · Polyethylene glycol · Bile salts · Urea

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Introduction

Enzymes are highly selective catalysts, able to operate under mild reaction conditions, properties that make them potentially attractive for industrial purposes. The biological function of lipases is mediated by an interaction between lipid and water interfaces that affects their activity [1]. Surfactant–enzyme interactions in aqueous solutions have been extensively studied for technical applications, such as drug delivery, cosmetics and detergency, and for studying interactions between membrane proteins and lipids [2], and then both components must be compatible with each other.

Lipases improve the washing capacity of the protease-containing detergents and improve the removal of fatty food stains and sebum from fabrics, which are difficult to remove under normal washing conditions [3]. Another important factor is that using hydrolytic enzymes (lipases, proteases and amylases) in detergent formulations allows laundering at lower temperatures, and thus reduces energy expenditure [4]. Accordingly, lipases must be characterized in terms of their stability towards surfactants [5–7].

Polyethylene glycol (PEG) is a synthetic polymer having low toxicity, soluble in aqueous solution, largely used in industrial biotechnological process [8].

Urea is a well-known protein denaturing agent [9] that can affect the enzyme structure by direct interaction with the macromolecule or by an indirect action through effects on the structure and properties of the surrounding solvent or by a combination of both of these mechanisms [10].

In this work we compared the effect of urea, PEG and investigated the influence of surfactant charge and chain length on the lipase activity of a lipase from *Pachira aquatica*.

Experimental Procedures

Materials

Pachira aquatica, known in Brazil as Munguba or Castanheira d'água, is a tree belonging to the Bombacaceae family, found in South America, and studies [11] developed on the composition of the seeds demonstrate that *Pachira aquatica* has a high oil content (44.1%) and could be a candidate for biodiesel production, so we were motivated to investigate the presence of a lipase that could be isolated from that source.

The seeds from *Pachira aquatica* were collected from trees of our Campus, at São José do Rio Preto, State of São Paulo. They were washed, peeled and extracted by homogenization in a food processor with a solution containing: 3 mM DTT, 1 mM EDTA, 10 mM sodium metabissulfite and 50 mM Tris–HCl buffer (pH 8.0) at 25 °C. The samples were clarified by centrifugation at 9,000×*g* by 40 min at 4 °C using a Jouan CR3i refrigerated centrifuge and the supernatant was concentrated and stored until use in liquid nitrogen. The purification was performed as described [12]. The lipase was purified 9.6-fold with specific activity of approximately 641 U/mL, showing one band in electrophoresis. The enzyme is a lipase as demonstrated in assays using soybean oil as substrate ($U > 1$). This lipase has a molecular weight of ~55 kDa and maximum activity at 40 °C and pH 8.0. The surfactants sodium dodecyl sulfate (SDS), sodium octyl sulfate (SOS), dodecyltrimethylammonium bromide (DoTAB), tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich Co., (USA) and recrystallized from ethanol and ethyl acetate. The reagents, Triton X-100, Tween 80 (Vetec, Brazil) and PEGs (Fluka, Switzerland) were used as provided.

Methods

Protein Concentration

Protein concentration was determined according to the method of Bradford [13], using bovine serum albumin (BSA) as standard.

Lipase Assay

We used a modified version of the procedure proposed by Chang et al. [14], based on the hydrolysis of *p*-NPA. The substrate was dissolved in 6% dimethyl sulfoxide (DMSO) and added to 30 mM Hepes buffer pH 7.5 and 0.264 U/mL of the enzyme. We had to choose this substrate because it is soluble in aqueous solutions, allowing to investigate the effect of added tensoactives. The assay mixture was incubated for 5 min at 25 °C and lipase activity was measured at 410 nm using a double-beam spectrophotometer Cintra 10e (GBC Scientific Equipment Pty Ltd), allowing to discount the spontaneous hydrolysis of *p*-NPA. Each assay was carried out in triplicate.

Effects of Surfactants

The effects of surfactants on lipase activity were studied by carrying out the assays at different surfactant concentrations (0–50 mM). We used SDS, SOS, taurocholic acid, sodium glycholate, CTAB, DTAB, TTAB, Triton X-100 and Tween 80.

Lipase stability was also analyzed by preincubating the enzyme with 10 mM of each surfactant, in the presence or not of 10 mM calcium chloride. Aliquots were withdrawn at intervals and used for the assay, from 0–180 min.

Effects of Polyethylene Glycol

The effects of PEG of various molecular weights (0.2, 8 and 12 kDa) were studied at a range concentration from 0 to 25 mM, 25 °C and pH 7.5. The enzyme stability was measured by preincubating the enzyme with a 10 mM concentration of the different MW PEGs and the test was performed at 25 °C and pH 7.5. Aliquots were withdrawn at intervals and used for the assay.

Effects of Urea

The effects of urea (0–50 mM) was determined at 25 °C and 30 mM Hepes buffer pH 7.5 by the assay methods described above. Lipase stability in the presence of 10 mM urea was analyzed by preincubating the enzyme, and aliquots were withdrawn at different intervals for assays, from 0 to 240 min.

Results and Discussion

Effects of Surfactants

As shown in Fig. 1a, SOS and SDS showed inhibitory effects in the whole concentration range. Taurocholic acid did not show a clear trend. Lipolytic activity increased initially and afterwards remained lower but very close to the 100% reference value. Glycholate induced an interesting response: increased lipase activity up to 10 mM (108% relative activity), but above 20 mM there was a decrease, obtaining similar values to those observed for SOS. The results observed for glycholate are similar to those reported [15] for a lipase from *Aspergillus carneus*.

SDS induced the strongest inhibition on lipase activity; at 50 mM, reduced approximately 40% the relative activity, probably due to a low critical micelle concentration (cmc) value or by influence its chain length.

Among the tested cationic surfactants (Fig. 1b), CTAB showed clear stimulatory effects, inducing a 32% activity increase in lipase activity. The effect seems to be proportional to the chain length (CTAB > TTAB > DTAB). This behavior can be also due to micelle formation, because CTAB cmc is low, facilitating the interaction substrate-enzyme. The lipase activity was also slightly increased (6%) by TTAB. On the other side, DTAB did not induce a clear response up to 20 mM, but caused a very weak inhibition that reached 4% at 50 mM.

In the presence of nonionic surfactant (Fig. 2) the lipase activity was inhibited for all the tested concentrations. However, above 20 mM, Tween 80 inhibition was more pronounced than that in the presence of Triton X-100. A similar inhibition produced by Triton X-100 was found also [16] for chicken pancreatic lipase.

A stimulating effect of surfactants on enzymatic hydrolysis has been reported several times. Explanations for the surfactant effect include an increase of enzyme stability and increasing accessibility of the substrate [17].

An interaction surfactant–protein involves some kind of protein denaturation. However, the reduced activity can include other explanations beyond conformational changes of the protein. Gargouri et al. [18] related the inhibition of pancreatic lipase activity by tensoactives to a desorption of the enzyme from its substrate occurring after a change in the interfacial quality.

In order to explain the correlation between the surfactant concentration and the decrease of enzymatic activity it can be conjectured that the active site is occupied by surfactant molecules when it reaches a certain concentration, or alternatively by interaction of the substrate molecules with surfactants.

Figure 3 shows that the only increase of activity was obtained for CTAB at 10 min of exposure, but a reduction of activity was evident after 20 min. Up to 1 h, inhibition

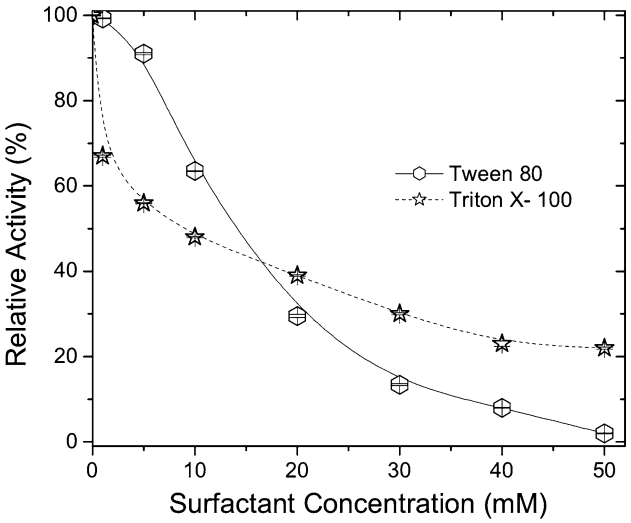

Fig. 2 Effect of nonionic surfactants concentration on lipase activity using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

was larger in the presence of Triton, followed by SDS and CTAB. After that time, the effect of Triton was lower than for SDS and CTAB.

Lipase stability also was tested in the presence of 10 mM $CaCl_2$ (Fig. 3). The enzyme showed greater stability to SDS and CTAB than in the absence of calcium, but the data obtained in the presence of Triton X-100 did not show significant differences. Ions Ca^{++} are known to provide a stabilizing effect on the 3D structure of any lipase but surfactant aggregates and interactions Ca^{++}-micelles compete favorably with enzymes for bound calcium ions [19]. Since Triton X-100 has a low value for cmc, this could explain the absence of significative differences of stability.

Effect of Polyethylene Glycol

Incubation of lipase with various concentrations of PEG 0.2, 8 and 12 kDa increased the hydrolytic activity in aqueous solution (Fig. 4) as described by Grimonprez and

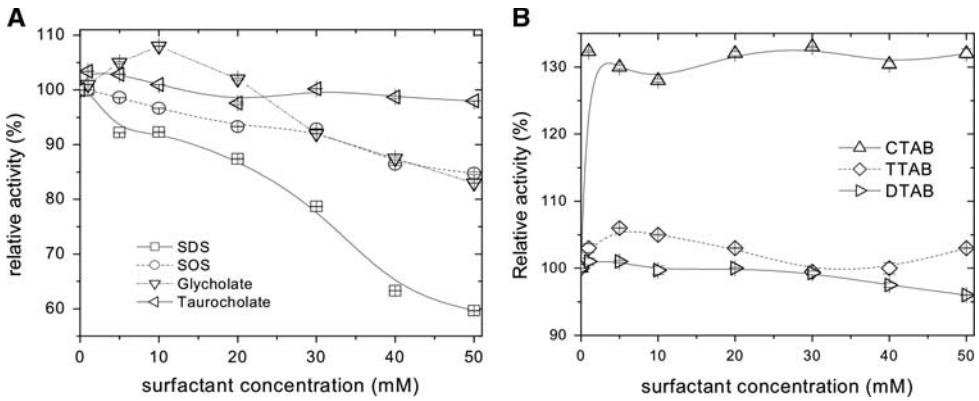

Fig. 1 Effect of anionic (**a**) and cationic (**b**) surfactants concentration on the activity of the lipase from *Pachira aquatica* using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

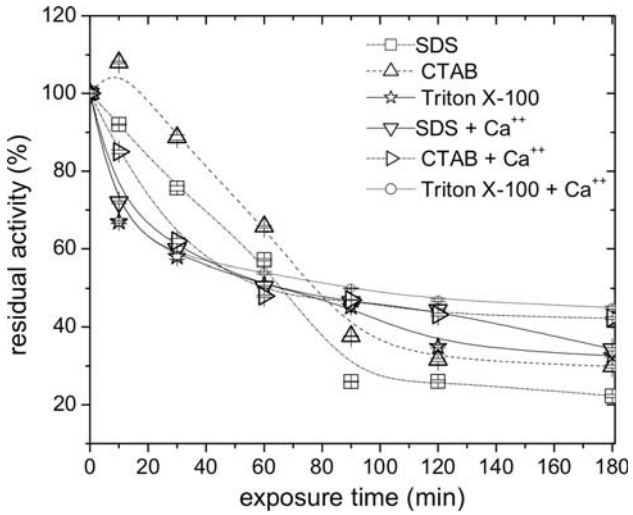

Fig. 3 Lipase stability in the presence of surfactants in the absence and in the presence of 10 mM $CaCl_2$. Assay performed using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

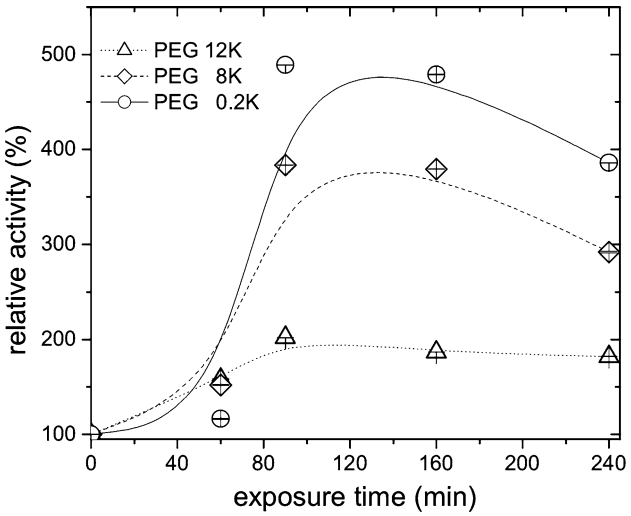

Fig. 5 Lipase stability in the presence of 10 mM PEG. The assays were done using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

Johansson [20] for phosphofructokinase and glyceraldehydes-3-phosphate dehydrogenase, Otero et al. [21] for a family of *Candida rugosa* isolipases and by Börjesson et al. [22] for lignase from *Trichoderma reesei*. The increase in activity was proportional to PEG molecular mass as described by Pancera [23] and Otero [21], and no inhibition was observed within the tested range, although the stimulation effect tends to decrease with time after an initial burst. This behavior can be due to an enhance in hidrophobicity promoted by increase in the chain length as suggested by Forciniti et al. [24] and then facilities the coupling of substrate in the active site or still by reduced water activity and favoring the exposure of hydrophobic residues in the opening lid, or even enzyme aggregation. A comparative analysis of crystallographic models of isolipases from *Candida rugosa* suggests a role for PEG in the enzyme activation and further stabilization of the actived form via dimerization in aqueous media [21].

The lipase showed stability in the presence of several PEGs maintaining its activity significantly higher than the control after 4 h (Fig. 5).

It is noteworthy that differently from the data shown previously (Fig. 4) the lipase stability in the presence of PEG was larger for PEG 200 and 8,000 than for 12,000

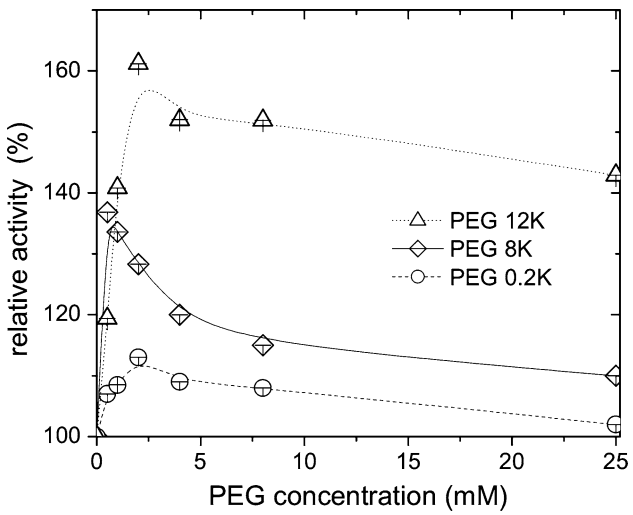

Fig. 4 Lipase activity in the presence of PEG. The assays were done using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

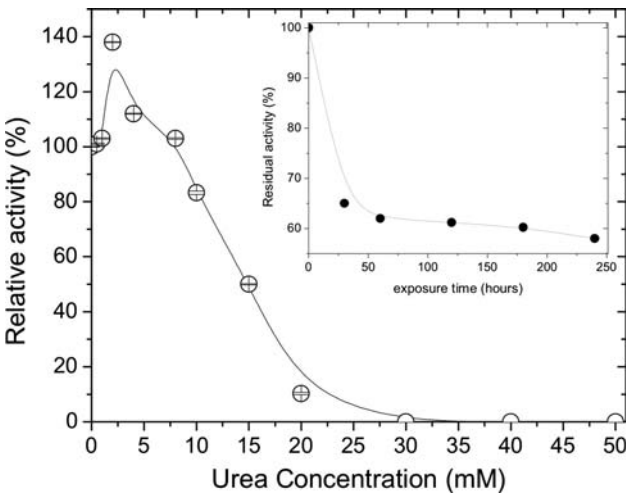

Fig. 6 Effect of urea concentration on lipase activity. *Inset*: lipase stability in the presence of 10 mM urea. The assays were done using *p*-NPA as substrate at 25 °C and pH 7.5. Each point is the average of three replicates ± SD

(Fig. 5). The results agree with those reported that indicate that when the molecular mass of PEG is larger than 1,000 g mol^{-1} the chains are excluded of bigger aqueous compartments around the protein; larger chains can dehydrate the protein [25].

Effect of Urea

Up to 10 mM we can observe an increase and afterwards an inhibition of lipase activity (Fig. 6). We attribute this effect to a conformational change (unfolding) caused by urea. In the presence of 10 mM urea (Fig. 6) the enzyme was inhibited, been nearly 40% of initial after 30 min of exposure to urea and remained so thereafter. At 240 min of exposure the relative activity was 58%.

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