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Study of a new spectrophotometric end-point assay for lipase activity determination in aqueous media

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ABSTRACT

A new spectrophotometric end-point method to evaluate lipase activity in aqueous media is described. This procedure is based on blocking the enzyme reaction by adding chloroform:isoamyl alcohol (24:1) as a denaturating agent, and removing the precipitate by centrifugation. Emulsifier screening showed that the chemical component with the least effect on lipase activity from *Thermomyces lanuginosus*, *Mucor javanicus*, *Aspergillus niger*, *Rhizomucor miehei*, *Penicillium camemberti* and *Burkholderia cepacia*, in the proposed method, was 0.1% (w/w) gum arabic. In contrast, Triton X-100, SDS, sodium cholate and Tween 80 reduced lipase activity, depending on the microbial source of the latter. A comparative study with other end-point methods based on the addition of chemical reagents (NaOH, Na_2CO_3, THAM or acetone −ethanol) or in thermal treatments (chilling or heating) was performed. The proposed procedure was shown to be more accurate in comparison with other methods that were tested. Furthermore, the effectiveness of the method was demonstrated in a study of the specificity of six commercial lipases toward *p*-nitrophenyl decanoate (C10) and *p*-nitrophenyl palmitate (C16). The study also analyzed the kinetic behavior and catalytic efficiency of lipase from *T. lanuginosus* and *B. cepacia* toward *p*-nitrophenyl decanoate and *p*-nitrophenyl palmitate.

1. Introduction

Lipases (triacylglycerolacylhydrolase; EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols to release free fatty acids and glycerol. These enzymes are effective catalysts for various inter-esterification and trans-esterification reactions in the presence of decreasing amounts of water and often in the presence of organic solvents (Foresti & Ferreira, 2010; Jaeger, Dijkstra, & Reetz, 1999). Their versatility means that lipases are the enzyme of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Singh & Mukhopadhyay, 2012). Recently, researchers reported that the synthesis of flavor esters for the food industry (Mahapatra, Kumari, Garlapati, Banerjee, & Nag, 2009), modification of triglycerides for the fat and oil industry (Fernandez-Lafuente, 2010), and resolution of racemic mixtures used in the synthesis of agro-chemical compounds (e.g., herbicides) (Tanaka, Yoshida, Sasaki, & Osano, 2002) can be performed with these enzymes.

One of the critical steps for effective implementation of enzymes in industry is the determination of the enzymatic activity of enzyme preparations (Lonsane & Ghidlyal, 1992). The selection of suitable reactions and substrates is crucial for an activity assay. So, a wide range of lipase assay methods differing in terms of their basic principle (kinetic or end-point), substrate selectivity, sensitivity and applicability have been developed (Beisson, Tiss, Riviere, & Verger, 2000; Gupta, Rathi, Gupta, & Bradoo, 2003; Hasan, Shah, & Hameed, 2009). Most of the methods are based on hydrolytic assays, which generally use *p*-nitrophenyl esters of various-chain-length fatty acids as substrates (e.g. *p*-nitrophenyl palmitate, *p*NP-palmitate) (Winker & Suckman, 1979; Pencreac'h & Baratti, 1996).

In particular, the kinetic method with *p*NP-palmitate as a substrate presents a major limitation to the low solubility of this substrate in an aqueous medium. In this regard, Palacios, Busto, Perez-Mateos, Pilar-Izquierdo, and Ortega (2011) reported non-reproducible results in measuring the lipase activity from *Thermomyces lanuginosus* and *Burkholderia cepacia* by kinetic methods, as a consequence of the complexity of evaluating the emulsion color of *p*NP over the time. In the case of an end-point methodology, the blocking of residual/unquenched enzymatic activity is essential, to avoid a false increase in color intensity and to maintain the reproducibility of the results (Kanwar, Kaushal, Jawed, Gupta, & Chimni, 2005). Different strategies have been described in the literature to block residual lipase activity, after a certain period of incubation, with *p*NP-palmitate as the substrate (Table 1). These

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Table 1
Review of the stop reagent/technique applied in end-point lipase assays using *p*NP-palmitate as substrate.

Emulsifying agent	[*p*NP-palmitate][a]	Reaction conditions (pH, *T*, *t*)	Stop reagent/technique	References
Triton X-100 and gum arabic	0.36	6.5, 40 °C, 5 min	2% THAM (1:1)[b]	Mayordomo et al. (2000)
Triton X-100	0.45	7.0, 37 °C, 1 h	1-Propanol (1:1)[b]	Yang et al. (2002)
None	6.5–6.8	4.4–9.2, 20–55 °C, 5 min	0.25 mol L^{-1} Na_2CO_3 (1:1)[b]	Chiou and Wu (2004) Ye et al. (2005) Ozmen, Sezgin, and Yilmaz (2009) Huang et al. (2009)
None	0.02–0.3	5.0–7.5, 20–55 °C, 15 min	0.25 mol L^{-1} Na_2CO_3 (2.5:1)[b]	Ozyilmaz (2009)
Triton X-100 and gum arabic	0.067–1	5.0–9.5, 30–70 °C, 5 min	0.25 mol L^{-1} Na_2CO_3 (1:1)[b]	Ozan, Ozyilmaz, Cokmus, and Caliskan (2009)
Triton X-100 and gum arabic	0.02–0.15	7.0, 37 °C, 5 min	0.10 mol L^{-1} Na_2CO_3 (volume no specify)	Yi et al. (2009)
Triton X-100	0.625	8.0, 55 °C, 30 min	0.1 mol L^{-1} ZnSO4 (1:40)[b]-immersing in ice for 5 min	Yoo et al. (2011)
None	0.84	7.5, 35 °C, 5 min	Ethanol 96% (1:10)[b]	Hlavsová et al. (2009)
Triton X-100 and gum arabic	0.24–0.79	8.0, 37–60 °C, 10 min	Putting the test tube in ice	Singh et al. (2009) Ji et al. (2010)
Sodium deoxycholate and gum arabic	0.76	8.0, 37 °C, 15 min	No specify	Gopinath, Hilda, Priya, and Annadurai (2002)
Triton X-100 and gum arabic	2.4	8.0, 37 °C, 30 min	No specify	Supakdamrongkul, Bhumiratana, and Wiwat (2010)
Triton X-100 and gum acacia	0.5	8.5, 55 °C, 10 min	No specify	Pahujani, Kanwar, Chauhan, and Gupta (2008)
Sodium deoxycholate and gum arabic	0.79	8.0, 37 °C, 15 min	No specify	Salihu, Alam, AbdulKarim, and Salleh (2011)

[a] Concentration in the reaction media (in mmol L^{-1}).
[b] Rate reagent to stop the reaction: reaction media volume.

end-point assays are based on the addition of reagents such as trishydroxymethyl aminomethane (THAM) (Mayordomo, Randez-Gil, & Prieto, 2000), 1-propanol (Yang, Koga, Nakano, & Yamane, 2002), Na_2CO_3 (Yi, Noh, & Lee, 2009), $ZnSO_4$ (Yoo et al., 2011) or ethanol (Hlavsová, Zarevúcka, Wimmer, Macková, & Sovová, 2009), or in thermal treatment of the samples by immersion in ice (Ji, Xiao, He, & Liu, 2010; Singh, Singh, & Banerjee, 2009) or heating.

On the other hand, lipases present very complex mechanisms of catalytic action, including the process of "interfacial activation" (Winkler, Darcy, & Hunziker, 1990). Access to the active site may be shielded by a mobile lid in a closed or an open position, which induces the inactive or the active conformation of the enzyme (Santarossa et al., 2005). Emulsifiers have been described that may prevent negative interaction between the hydrophobic face of the lid with the water (Fernandez-Lorente, Palomo, Cabrera, Fernandez-Lafuente, & Guisan, 2007). The emulsifiers may shift the close/open equilibrium between the forms of lipases toward the open form in aqueous homogenous media in the process (Nobel, Cleasby, Johnson, Egmond, & Frenkel, 1993). However, the emulsifiers may also act as inhibitors or deactivating agents (Bano, Gonzalez-Navarro, & Abad, 2003). These findings suggest the need to determine the most suitable emulsifier for each particular reaction (Fernandez-Lorente et al., 2007).

In the current work, we propose a new end-point method based on blocking the residual lipase activity by precipitating the enzyme from the reaction mixture. The method is based on the denaturation of the enzyme with a mixture of chloroform and isoamyl alcohol and the removal of the denatured protein by centrifuging (Staub, 1965). Firstly, the effect of different emulsifiers on the activity of several commercial lipases evaluated by the proposed method was studied. After selecting the best emulsifier, the effectiveness of the method was demonstrated by comparison with other end-point spectrophotometric methods described in the literature using *p*NP-palmitate. Finally, the reproducibility and utility of this spectrophotometric end-point assay was demonstrated by determining the substrate specificity and catalytic efficiency of lipases from different microbial sources.

2. Materials and reagents

2.1. Material and reagent

Six lipases from different sources were purchased from Sigma–Aldrich Corporation (St. Louis, Mo, USA): Amano lipase A from *Aspergillus niger*, Amano lipase PS from *B. cepacia*, Amano lipase G from *Penicillium camemberti*, Amano lipase M from *Mucor javanicus*, the enzyme preparations of Novozymes Palatase from *Rhizomucor miehei*, and Lipolase from *T. lanuginosus*. Triton X-100, Tween, 80, gum arabic, sodium cholate, *p*NP-decanoate, *p*NP-palmitate and *p*NP were purchased from Sigma–Aldrich Corporation. All other chemicals used in the present study were of analytical or better grade without further purification.

2.2. Lipase assay

Lipase activity was measured spectrophotometrically using an assay based on the hydrolysis of *p*NP-palmitate, at 1.5 mmol L^{-1}. The reaction mixture consisted of 3.5 mL of 50 mmol L^{-1} tris(hydroxymethyl)aminomethane (Tris)–HCl buffer (pH 8.0) containing 1 g L^{-1} gum arabic and 0.4 mL of 15 mmol L^{-1} *p*NP-palmitate dissolved in 2-propanol. The mixture was prewarmed at 40 °C, and then 0.1 mL of enzyme solution was added. After 5 min of incubation at 40 °C the reaction was stopped by adding 1.5 mL of Marmur solution (chloroform:isoamyl alcohol, 24:1) (Marmur, 1961). The sample was centrifuged at 10,000 rpm for 5 min at 4 °C and the clear supernatant (aqueous phase), was taken off. The optical density of supernatant was then measured at 410 nm. The molar extinction coefficient of *p*NP under these conditions (0.0048 mol^{-1} L cm^{-1}) was estimated by using a standard curve of *p*NP in 2-propanol (ranging from 10 to 360 μmol mL^{-1}) treated with the Marmur solution. Controls in which the enzyme solution was substituted by 50 mmol L^{-1} Tris–HCl buffer (pH 8.0) containing 1 g L^{-1} gum arabic were assayed in all cases to deduct any non-enzymatic activity. One unit of activity (U) was defined as the amount of enzyme that liberates 1 μmol of *p*NP per minute under the assay conditions. The activity of lipase against *p*NP-decanoate