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Development of a high-throughput liquid state assay for lipase activity using natural substrates and rhodamine B

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ABSTRACT

A fluorescence-based assay for the determination of lipase activity using rhodamine B as an indicator, and natural substrates such as olive oil, is described. It is based on the use of a rhodamine B–natural substrate emulsion in liquid state, which is advantageous over agar plate assays. This high-throughput method is simple and rapid and can be automated, making it suitable for screening and metagenomics application. Reaction conditions such as pH and temperature can be varied and controlled. Using triolein or olive oil as a natural substrate allows monitoring of lipase activity in reaction conditions that are closer to those used in industrial settings. The described method is sensitive over a wide range of product concentrations and offers good reproducibility.

Enzymatic reactions often surpass chemical transformations in terms of cost, product yield, reaction conditions, specificity, and efficiency. Furthermore, enzymes are biodegradable, whereas many chemical catalysts are not. Enzymes' advantages are recognized in several industrial sectors, and as a consequence the enzyme market is predicted to reach $6 billion (USD) by 2016 [1]. Lipases (EC 3.1.1.3) are one of the most important industrial biocatalysts [1,2]. They catalyze a variety of reactions using a vast array of substrates. However versatile they may be, lipases are primarily tailored for the hydrolysis of acyl glycerides [3]; their ability to hydrolyze ester bonds and release glycerol and fatty acids confers these enzymes a huge potential for industrial applications [4,5]. The development of novel applications for lipases, however, is highly dependent on the identification of enzymes with desired properties, which requires precise biochemical and biophysical characterization of these catalysts [6].

Lipase hydrolysis is commonly measured via colorimetric assay using *para*-nitrophenyl esters (*p*NP-esters) as substrates. The advantages of this method over others such as titrimetric analysis include its rapidity and simplicity [7]. Unfortunately, *p*NP-esters are not natural substrates of lipases, nor are they the intended industrial target, and they often require the use of organic solvents [8,9]. For a qualitative approach with natural substrates, a rhodamine B (RhB) plate assay with olive oil has been widely used for the identification of microbial lipases [10,11]. Activity leads to hydrolytic halos, which contain released fatty acids and are revealed under ultraviolet (UV) light.

The use of RhB for the quantitation of lipase activity has been described previously by Jette and Ziomek [9]. Lipase hydrolytic activity was calculated on the basis of fluorescence emission observed when RhB interacts with released fatty acid. This assay, however, involves solid reaction media (agar), which makes this method complicated and less flexible than a liquid-based approach. Using solid media also decreases reproducibility because of the slow enzyme diffusion rate through a rhodamine–triglyceride–agarose plate gel [9]. Furthermore, preparing multi-well gel agarose plates as a means to increase throughput requires numerous complex steps. In addition, some steps cannot be performed by an automated pipetting station, impairing its use for high-throughput screening experiments.

In this article, we propose an improved fluorescence-based assay for quantitative lipase activity detection using natural

Abbreviations: *p*NP-ester, *para*-nitrophenyl ester; RhB, rhodamine B; UV, ultraviolet; *p*-NPC18, *p*-nitrophenyl stearate; *p*-NP, *p*-nitrophenol; LipAT, lipase from *Aneurinibacillus thermoaerophilus*; EstGtA2, carboxylesterase from *Geobacillus thermodenitrificans*; RhB–OOe, rhodamine B–olive oil emulsion mixture; RhB–TOe, rhodamine B–triolein emulsion mixture.

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triglycerides as substrates that can be carried out in liquid format using 96-well plates. Toward this goal, we have prepared a stable emulsion using triolein or olive oil, RhB, and gum arabic that is easily pipetted and fully compatible with a high-throughput format. Fatty acids that are released via lipase action interact with RhB, leading to fluorescence intensity changes measured with a plate reader.

Materials and methods

Chemical and reagents

RhB, gum arabic from the acacia tree, sodium phosphate, triolein, *p*-nitrophenyl stearate (*p*-NPC18), *p*-nitrophenol (*p*-NP), and oleic acid were purchased from Sigma–Aldrich. Olive oil was obtained from Bertolli (Gentile extra virgin) purchased in a local grocery. Commercial lipase A was obtained from Buckman North America. Lipase from *Aneurinibacillus thermoaerophilus* (LipAT) and carboxylesterase from *Geobacillus thermodenitrificans* (EstGtA2) were produced and purified in our laboratory using a recombinant DNA strategy [12,13].

Liquid reaction mixture preparation

A Rhodamine B–olive oil (or triolein) emulsion mixture (RhB–OOe or RhB–TOe) was prepared using 20 mM sodium phosphate (pH 7), 5% (w/v) gum arabic, 0.001% (w/v) RhB, and 2.5% (v/v) olive oil or triolein. The mixtures were emulsified with a DrinkMaster for 5 min, and then the pH was adjusted. Fluorescence spectra of emulsified solutions with and without lipase were recorded to verify the maximum emission wavelength for monitoring fatty acid release. The emulsions were stored at 4 °C away from light to avoid fluorophore bleaching. Fluorescence of the emulsion was monitored over 2 months to ensure that the emulsion and fluorescence yield were stable.

Activity assay and fatty acid standard curve

Activity assays were performed using different quantities (4.6–46.5 μg) of commercial lipase A and two recombinant enzymes (lipase LipAT and carboxylesterase EstGtA2 prepared in our laboratory [13]). The enzymatic assays were performed in 96-well plates at pH 7 and 25 °C using a BioTek Synergy Mx microplate reader. The enzymatic activities of lipase A and LipAT were performed at different temperatures (25, 45, and 65 °C) at pH 7 and at different pH values (5, 7, and 9) at 25 °C. The enzymatic reactions were initiated by adding 2 μl of enzyme solution to 200 μl of emulsion. The rate of hydrolysis was calculated from fluorescence emitted at 580 nm (excitation wavelength = 350 nm) with a gain of 50. Emulsions without enzyme, and RhB–gum arabic mixture with enzyme but without substrate, were also used as negative controls. Standard curves were constructed following changes in fluorescence intensity after the addition of increasing amounts of oleic acid (3–50 mM) to the RhB–gum arabic solution. The pH and temperature were adjusted for each standard sample. The fluorescence intensity measurements were performed using exactly the same conditions as those used for the activity assay. For reproducibility and precision, the sample preparations and measurements were carried out in triplicate. Polynomial equations were used to convert fluorescence emission changes into the hydrolysis rate. One unit of activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per minute per mg of protein under our assay conditions.

Colorimetric assay using pNP-ester

Lipase specific activity was determined spectrophotometrically by measuring the amount of *p*-NP released after hydrolysis of *p*-NPC18. The substrate was prepared in acetonitrile. The standard reaction (0.2 ml) contained 100 μM *p*-NPC18, 10% (v/v) acetonitrile, 20 mM sodium phosphate buffer (pH 7), and 0.4 μg of enzyme. Gum Arabic (0.01%, w/v) was added to promote emulsification of *p*-NPC18. The reaction was performed in 96-well microplates. The production of *p*-NP was monitored at 405 nm using a Biotek Synergy Mx microplate reader. The extinction coefficient was determined using a known amount of *p*-NP under various colorimetric assay conditions in order to quantify the amount of *p*-NP released during the enzymatic reactions.

Specific standard curves using a known amount of *p*-NP were measured under the same conditions as those used for enzymatic reactions. One unit of activity was defined as the amount of enzyme releasing 1 μmol of *p*-NP per minute under our assay conditions.

Results and discussion

Determination of lipase activity using fluorescence from RhB olive oil emulsions

For this assay, RhB emulsified mixtures with and without olive oil were prepared as described in Materials and Methods. The stability of RhB–OOe (emulsified mixture) was monitored by measuring its fluorescence intensity. We found that the emulsified mixture can be stored at 4 °C for up to 2 months without any significant intensity change. All emulsions studied were excited at 350 nm (which corresponds to the typical wavelength emitted by a UV transilluminator) and showed maximal fluorescence emission at 580 nm. Background fluorescence values from the RhB–gum arabic solution and RhB–OOe were recorded. It is important to note that RhB–gum arabic solutions emit fluorescence significantly under our conditions, but this fluorescence was quenched when olive oil was added to the RhB–gum arabic mixture (data not shown). Quenching was relieved and fluorescence intensity increased when olive oil was hydrolyzed, releasing fatty acid and glycerol as shown in Fig. 1A. Fatty acids and glycerol, however, did not emit fluorescence at 580 nm when measured in the absence of RhB (data not shown).

Different quantities of lipase A, ranging from 4.6 to 46.5 μg, were used with RhB–OOe to measure lipase time-dependent lipase-catalyzed hydrolysis at 25 °C and pH 7 (Fig. 1A). As expected, hydrolysis of olive oil resulted in increased fluorescence intensity. As shown in Fig. 1A, fluorescence intensity was correlated with the quantity of enzyme used. No increase in fluorescence intensity was observed when carboxylesterase EstGtA2 enzyme was used with the emulsion because this enzyme is unable to hydrolyze olive oil [13]. The same results were observed for RhB–OOe without enzyme and for RhB with enzyme but without substrate (data not shown). Standard curves for oleic acid in the presence of RhB and gum arabic were prepared, and a linear regression was performed (Fig. 1B), allowing the calculation of lipase activity. Quantification of the amount of fatty acid released after enzymatic hydrolysis was calculated (Fig. 1C) using the standard curve generated using oleic acid. We recognize that when using a natural substrate such as olive oil, which may release various mixtures of fatty acids on its hydrolysis (depending on the enzyme specificity), it is difficult to construct a representative standard curve that reflects the *precise* amount of *each* fatty acid released, correlated with lipase activity. Various mixtures of linoleic, oleic, and other fatty acids, for instance, might not lead to the same exact change in polarity in the medium and, consequently, in fluorescence intensity. It is worth