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Rapid determination of the synthetic activity of lipases/esterases via transesterification and esterification zymography

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HIGHLIGHTS

- A new and versatile method to determine synthetic activity by zymography is proposed.
- Specific detection of proteins which perform the synthetic activity desired.
- Zymogram results are confirmed by titration, GC-analysis and NMR.
- This method may be applied for enzyme high-throughput screening.

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ABSTRACT

A new simple and extremely versatile zymography method based on transesterification and esterification reactions was developed in this work. The method consists in building a transesterification or esterification reaction medium according to the goal of the research. Since commercial enzymes and crude extracts commonly consist in *pools* of proteins, this methodology provides a means to determine which protein are responsible for the enzymatic activity desired. The protocol may be potentially used as a high-throughput screening of lipases or esterases that catalyzes the synthesis reaction for biodiesel and biolubricants production.

1. Introduction

The environmental interest that surrounds the world leads to a chemistry (i.e. green chemistry) in which the enzymes may have special relevance. In this context, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) should be highlighted. *In vivo*, these enzymes catalyze triglycerides hydrolysis into free fatty acids and glycerol at the oil–water interface [1]. *In vitro*, and in the absence of water, several synthetic reactions can be catalyzed by lipases such as acidolysis, aminolysis, alcoholysis, esterification, transesterification and interesterification [2]. Synthetic reactions catalyzed by lipases, such as esterification and transesterification, have earned importance due to industrial applications of the bioproducts that come from then [3], such as biodiesel [4,5] or biolubricants [6].

Several studies have reported the industrial lipase applications for transesterification and esterification reactions [7–11] and also its enzymatic synthesis could obtain more successful results than chemical catalysis [12].

The polyacrylamide gel electrophoresis (PAGE) is very widespread to separate proteins by their electrophoretical mobility. Zymography comprises a rapid and practical method which applies this PAGE on detection of enzymes responsible for the desired activity [13]. Thus, its use presents some advantages such as: (a) reducing timeframe for obtaining results; (b) identify a desired enzyme activity among a pool of proteins/enzymes found in crude extracts from non-commercial and commercial sources.

Zymography has been largely used for detection of enzymes which play proteolytic reactions [13,14] and for lipases and

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esterases detection in both hydrolytic [15], and less frequently in synthetic reactions [16]. This method is based on staining the protein band responsible for the desired activity usually by precipitation of a reaction product.

The use of this technique for detection of transesterification activity has not been reported until the present date. Thus, the aim of this work is to describe one new, quick, and simple method that allows the detection of enzymes able to catalyze transesterification reactions, such as the biodiesel and biolubricants production [7,12]. The methodology could be useful in high-throughput screening of lipases/esterases, permitting the identification of the enzyme/s that is/are the real responsible species for the detected activity, as this should be coupled to a protein band in the PAGE experiment, allowing even possible future identification, extraction and purification of specific lipases/esterases.

2. Material and methods

2.1. Materials

The pre-stained molecular weight SeeBlue® Plus 2 was obtained from Novex by Life Technologies (PMW). Lipase AY Amano 30 (AY) was purchased from Amano Enzyme Inc. (Japan). Lipozyme TL 100L (TL) was provided by Novozymes S/A (Denmark). Lipomod 34 MDP™ (MDP) was from Biocatalysts Inc. (UK) and Lipase from *Pseudomonas* sp. type XIII (XIII) was purchased from Sigma Aldrich.

An enzyme preparation previously reported by our group, obtained by solid-state fermentation of babassu cake using a mesophilic strain of *Rhizomucor miehei* (IDAC accession number 071113-01), has been also used. This enzyme preparation consists of a dry fermented solid (DFS) prepared as described by Aguieiras *et al.* [7]. The crude enzyme extract was obtained from DFS extraction with phosphate buffer (0.1 mol L^{-1}, pH 7.0) as described by Gombert *et al.* [17]. This extract was used in zymography assays. The oleic acid was purchased from VETEC, Sigma Aldrich (Brazil), the trimethylolpropane (TMP) from Sigma Aldrich (Brazil) and the methyl esters from castor oil (castor biodiesel esters) were provided by PETROBRAS (Rio de Janeiro, Brazil). All enzymes were diluted to an appropriated concentration according to their specific activity in 5 mM phosphate buffer, pH 7.0.

2.2. Methods

2.2.1. SDS–PAGE experiments

Enzyme samples at 4–10 μg/mL were loaded in 12 % running and 4 % stacking PAGE gels. Sample protein concentration was determined according to Bradford's method [18] to be diluted in Laemmli's sample-loading buffer [19] excluding β-mercaptoethanol and containing bromophenol blue and SDS. Then the sample was applied to the stacking gel, without boiling, aiming to be comparable with zymogram gels. The electrophoretic running gel was performed at 180 V at room temperature until the end of the running gel. The gel was stained with Coomassie Brilliant Blue to identify proteins bands. The negative controls (blank) were prepared by boiling the enzyme aliquots diluted in sample buffer for 30 min before being applied into the gels, respectively with analogous non boiled enzymes. The preparation of each gel was repeated at least five times, in order to secure the reliability and reproducibility of this methodology.

2.2.2. Zymography for the specific transesterification and esterification activity

The same SDS–PAGE procedures described above were performed without staining. All zymograms presented in this work were performed on partially denaturing condition after being subjected to the reactions preliminarily in both native and half-denaturing conditions. We tested the PAGE-native for all conditions (data not shown), but we chose the SDS–PAGE, due to the higher resolution of the bands. After the electrophoretic run, gels were soaked for SDS removal with 1 % Triton X-100 in 5 mM phosphate buffer pH 7.0, for 20 min. Subsequently, they were washed with distilled water and then re-equilibrated in 5 mM phosphate buffer pH 7.0 for 10 min. Finally, the gels were submerged in three different reaction media which were prepared according to the final activity desired. For esterification activity, the medium was composed of ethanol and oleic acid in a molar ratio of 1:1. For biolubricant reaction, the medium consisted of oleic acid and TMP, in 3:1 M ratio. The third reaction consists of biodiesel obtained by castor biodiesel esters and TMP in a molar ratio of 4.5:1 [12]. For all reaction medium, the substrates were blended together and the gels subjected to each reaction. They were left stirring in a shaker in these reaction mixtures at a minimum rotation (approximately 50 rpm) at 45 °C for 24 h when TMP is used for biolubricant reaction and 3 h in the case of ethanol for biodiesel reaction, however, the time can vary according to the enzyme activity. The reactions were carried out until the appearance of the product deposition. Subsequently, the gels were washed and conditioned with acetic acid 1 % until image acquisition.

2.2.3. Esterification and transesterification reaction in bioreactors

Esterification reactions were carried out in 20 mL bench-scale reactors magnetically stirred (200 rpm) and thermostatized at 45 °C. The reaction medium was composed of oleic acid and TMP in molar ratio of 3:1 and 0.1 % of the commercial lipases MDP e XIII (w/w). Esterification activity was determined by oleic acid consumption in the esterification reactions with TMP, measured by automatic titration using triplicate samples of 100 μL removed from the reaction medium, mixed with acetone/ethanol 1:1, and using NaOH 40 mM as titrating reagent with an end point pH at 11. The acid oleic conversion was expressed by percentual of oleic acid consumed, from difference between the acidity at the final and at the beginning of the reaction.

In order to confirm the biodiesel and biolubricants production showed in zymogram bands, esterification and transesterification reactions were performed as described above:

Esterifications reactions:

(a) oleic acid and ethanol (molar ratio 1:1) using 20 % (w/v) DFS and
(b) oleic acid and TMP (3:1) with commercial lipase 4 % (w/v) 34 MDP.

Transesterification reactions:

(c) castor biodiesel esters and TMP (4.5:1) utilizing commercial lipase 4 % (w/v) 34 MDP.

The same conditions described in Section 2.2.2 were used. The products of "a" were analyzed trough GC analysis and the products of "b" and "c" were submitted to NMR analysis.

2.2.4. Product identification by nuclear magnetic resonance

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) was used to identify the structure and purity of synthesized compounds, using Agilent INOVA-300 (7,05T) spectrometer device. Samples were prepared in deuterated chloroform ($CDCl_3$) at 25 °C, at 5 % (1H) e 20 % ^{13}C concentration, and compared with pure chemical compounds spectra.