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A pectin–lipase derivative as alternative copolymer for lipase assay

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ABSTRACT

In this study Arabic gum and free lipase were successfully replaced by a lipase immobilized onto pectin (PECp-lipase) for pNP palmitate hydrolysis. Using a Central Composite Rotatable Design the optimum pH and temperature for free and PECp-lipase reaction were established at pH 8.0, 30–40 °C, and pH 8.0, 40–50 °C, respectively. PECp-lipase maintained 100% of initial activity after 35 days of storage at room temperature. The thermal kinetic parameters (k_d and $t_{1/2}$) and E_d evidenced that immobilization provide higher thermal stability to PECp-lipase compared to free enzyme. Thermodynamic parameters (ΔH°, ΔS° and ΔG°) confirmed the thermal stability acquired by PECp-lipase and indicated that tridimensional structure was preserved. The apparent Michaelis constant estimated for the PECp-lipase (1.15 mM) was not statistically different from the free enzyme (1.09 mM). PECp-lipase represents a faster, single step and, therefore, a very attractive substitute for the lipase standard methodology of pNP palmitate hydrolysis.

1. Introduction

Pectins comprise a complex family of heteropolysaccharides that play an important role in plant growth and development [1–3]. Currently, pectins represent one of the most studied polysaccharides as they are target of many applications in the food and pharmaceutical industry, such as their use as thickeners, stabilizers, gelling agents and texture modifiers [1,4,5].

Recently, the pectin extracted from the fruit of *Solanum lycocarpum*, known as lobeira in Brazil, was characterized as high methoxyl pectin with intrinsic viscosity and molecular weight similar to those observed in the citrus pectin [3]. However, *S. lycocarpum* pectin presented remarkable features, such as a two-fold higher emulsifying capacity than that presented by citrus pectin. This property allows new applications for *S. lycocarpum* pectin, making it an important key for many industrial processes.

Lipases are enzymes that catalyze the hydrolysis of acylglycerides and other esters at the interface between water and lipidic substrates [6–8]. Considering the hydrophobic nature of the substrate and the hydrophilic nature of lipase, it is mandatory for measurement of lipase activity the use of emulsifying agents that act in the interface between substrate and enzyme [9,10]. The standard colorimetric method for lipolytic activity measurement is based on the p-nitrophenyl palmitate (pNPP) hydrolysis [11–14]. To measure activity by this approach, two solutions are required: the p-nitrophenyl palmitate and Arabic gum solution. These solutions must be mixed cautiously and gently in order to properly emulsify the substrate, which makes this methodology laborious and time consuming.

Therefore, any other methodology employed to reduce the time spent preparing the substrate would provide a more rapid lipolytic reaction. In this matter, the use of immobilized lipase on a material presenting emulsifying capacity, such as the one presented by *S. lycocarpum* pectin, may be promising and, thus, decreasing the time spent during the reaction preparation. Moreover, previous reports have described improvements in the characteristics and activity of enzymes after immobilization, especially those related to thermal, pH and storage stability. In addition, immobilization processes usually allows the repeated use of enzymes, which may be of financial interest [15–18].

In this sense, in the present study lipase was immobilized onto the pectin extracted from *S. lycocarpum* and this pectin–lipase system was used to replace Arabic gum in the lipolytic reaction medium. This new system was evaluated regarding to the enzyme stability during storage as well as parameters such as thermal stability, thermodynamics and kinetics.

2. Materials and methods

2.1. Immobilization of lipase onto pectin from S. lycocarpum

The pectin from *S. lycocarpum* (PEC) was extracted as described by Torralbo et al. [3] and used as a support for the lipase immobilization (*Thermomyces lanuginosus* – Lipolase®, Novozymes, Araucária, Paraná, Brazil). The immobilization was carried out according to Silva-Filho et al. [19]. Briefly, the pectin was acti-

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vated using 0.1 mol L^{-1} sodium metaperiodate solution (PECp) and the immobilization of lipase was conducted by adding 1 mL of an enzyme solution (488 U), prepared in 0.1 mol L^{-1} Tris–HCl buffer, pH 8.0–5 mg of PECp. The system was incubated for 30 min at 4 °C under orbital stirring. Then, cold ethanol (92%, v/v) was added to precipitate the PECp-lipase. The precipitate product was washed twice with 0.1 mol L^{-1} Tris–HCl buffer to remove unbounded enzyme.

The scheme representing the mechanism of PECp-lipase production was obtained using the software ChemStetch 11.02 (Advanced Chemistry Development Inc., Toronto, CA). The tridimensional model of lipase was obtained using the software Jmol 13.0.6 (http://www.jmol.org).

2.2. Storage stability

The storage stability of the PECp-lipase was evaluated as follows. Samples were dried at 40 °C in an air-forced oven until constant weight. The dry samples were stored in hermetically closed vials at room temperature. The free lipase (solution containing 445.8 U) was also stored at room temperature and at 4 °C. The storage stability of the free lipase and PECp-lipase was determined by measuring their residual activity every 7 days.

2.3. Determination of enzyme activity

In this study, the methodology described by Babu and Rao [13] was employed for measurement of free lipase activity. Briefly, the production of p-nitrophenol (pNP) was monitored by its absorbance at 410 nm. A calibration curve was constructed in order to calculate the enzyme activity using the absorbance of standard pNP solutions. One unit of enzyme (U) was defined as the amount of free or immobilized lipase that releases 1 μmol min^{-1} mL^{-1} of pNP.

In order to evaluate the efficiency of Arabic gum substitution, two assays were carried out as follows:

Assay (A): the methodology described by Babu and Rao [13] was employed, but the Arabic gum was replaced by 0.1% of pectin. Lipase was added as free enzyme.
Assay (B): the reaction was performed using only PECp-lipase as emulsifying and lipolytic agent, in absence of Arabic gum.

The following equation was used to calculate the specific activity (U mg^{-1} protein) of the free and immobilized lipases:

$$\text{Specific activity} = \frac{\text{activity of free or immobilized lipase}}{\text{amount of protein}}$$

The amount of protein was determined according to methodology described by Bradford [20], using bovine serum albumin as standard.

The hydrolysis of olive oil was carried out according to Soares et al. [21]. Briefly, the substrate was prepared by mixing 2.5 mL of olive oil with 2.5 mL of Arabic gum solution (7% (w/v) in 50 mmol L^{-1} Tris buffer pH 8.0). Five milliliters of this substrate emulsion was added to the reactor containing 200 mg of PECp-lipase and the reaction proceeded at 40 °C for 60 min under stirring. The released fatty acid was titrated with 0.02 mol L^{-1} potassium hydroxide solution. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of free fatty acid/min under the assay conditions, calculated according following equation:

$$\text{EU} = \frac{(V_1 - V_2) \cdot M \cdot 106}{t \cdot m}$$

where V_1 represents the volume (mL) of KOH used in the titration of olive oil after hydrolysis; V_2 represents the volume (mL) of KOH used in the titration of olive oil before hydrolysis; M is the molar concentration (mol L^{-1}) of KOH; t is the reaction time (min); and m is the amount (mg) of PECp-lipase used.

2.4. Effect of pH and temperature on the enzyme activity

The effect of temperature and pH on the activity of the free and immobilized lipase was evaluated by using a Central Composite Rotatable Design (CCRD) 2^2 associated with Response Surface Methodology (RSM). For the experimental design, the two independent variables were used, both represented in two levels: for temperature, it was used 25 °C (low level) to 75 °C (high level); for the pH, it was used 3 (low level) to 9 (high level). A central point (50 °C; pH 6), with two replicates, was also included for statistical evaluation (at 95% confidence level).

Results from CCRD were analyzed using the software Statistica 6.0 (Statsoft Inc., Tulsa, USA, 1997). The adjustment of the experimental data for the independent variables in the RSM was represented by the second-order polynomial equation:

$$y = \beta_0 + \sum_j \beta_j x_j + \sum_{i<j} \beta_{ij} x_i x_j + \sum_j \beta_{jj} x_j^2 + e$$

where y is the dependent variable to be modeled; β_0, β_j, β_{ij} and β_{jj} are regression coefficients, x_i and x_j are independent variables and e is the error. The model was simplified by dropping terms that were not statistically significant ($p > 0.01$) by ANOVA.

2.5. Thermal behavior and inactivation kinetics of free and immobilized lipase

The thermal stability of free and immobilized lipase (PECp-lipase) was evaluated by measuring the remaining activity of pre-incubated free enzyme or PECp-lipase at different temperatures (25–75 °C) in Tris–HCl buffer solution (0.1 mol L^{-1}, pH 8.0) for 2 h. After 30 min at 25 °C (temperature equilibration), their activities were established as above described. The percentage of remaining activity was calculated as follows:

$$\text{Remaining activity} = \frac{\text{enzyme activity after incubation}}{\text{enzyme activity at the optimal temperature}} \times 100$$

Kinetic data analysis of thermal inactivation of enzymes can often be described by the first-order reaction [22–24]:

$$\frac{dA_t}{dt} = -k_d \cdot A_t$$

where A_t is the enzyme activity at treatment time t, and k_d is the reaction rate constant at the temperature studied.

For constant extrinsic/intrinsic factors, in the case of a first-order reaction, the kinetics can be described by the following equation:

$$\frac{A_t}{A_0} = e^{-k_d t}$$

where A_t is the enzyme activity at time t, A_0 is the initial enzyme activity, t is the treatment time (h), and k_d is the inactivation rate constant at the temperature studied. The inactivation rate constant k_d can be estimated by linear regression analysis of the natural logarithm of residual activity versus treatment time.