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Influence of the functional activating agent on the biochemical and kinetic properties of *Candida rugosa* lipase immobilized on chemically modified cellulignin

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Abstract

Candida rugosa lipase was immobilized by covalent binding on wood cellulignin (*Eucaliptus grandis*) chemically modified with different activating agents as carbonyldiimidazole, glutaraldehyde and sodium metaperiodate. The resulted immobilized derivatives were evaluated in both aqueous (hydrolysis) and organic (ester synthesis) media. In aqueous media a comparative study between free and immobilized derivatives was provided in terms of pH, temperature and kinetic constants (V_{max} and K_m) following the hydrolysis of *p*-nitrophenyl palmitate, in which new optima values were established. The experimental results suggested that functional activating agents render different interactions between enzyme and cellulignin, producing consequently alterations in the optimal reaction conditions. Different behavior was found when the immobilized derivatives were tested in organic media, under these conditions similar esterification activities were observed, independent on the agent used to active the immobilizing support. Reasons for this are discussed on the light of the interactions among the support, functional activating agent and lipase structure.

Keywords: *Candida rugosa lipase*; Cellulignin; Functional activating reagent; Immobilization

1. Introduction

Enzymes are versatile biocatalysts, capable of catalyzing diverse and unique reactions that are highly specific, often stereo specific, in their catalytic mechanisms, enabling simplified steps toward structurally specific product formation and making them highly desirable for targeted reactions [1]. Advances in biotechnology, in recent years, have made more efficient generation of specific enzymes available, expanding their potential, practical use in large-scale conversion of chemicals and materials [2–4].

Enzymes immobilized by water-insoluble supports can serve as reusable and removable catalysts, which often possess improved storage and operational stability [5]. Both chemical and physical methods have been developed for the purpose of immobilizing enzymes. Enzymes can be adsorbed onto inert solids, ion-exchange resins, or physically entrapped/encapsulated in solids, such as crosslinked gels, microcapsules, and hollow fibers. Enzymes can be covalently bonded to solids via various chemical bonding methods, such as cross linking, multi-functional reagents, or surface reactive functional groups [5,6].

Among these methods, chemical covalent bonds offer the strongest links, and thus the most stable enzyme-solid complexes [7]. To chemically bond enzymes to a solid, the structures and functions of both the enzymes and the solids should be considered. It is of utmost importance to consider the functional groups on the enzyme proteins through which the covalent bonds are formed and the physical and chemical characteristic of the support material onto which chemically reactive groups are to be attached [7,8].

Enzymes are protein molecules with wide ranging compositions and structural complexities. Enzyme proteins may consist of more than 20 types of amino acids. The functional groups on enzyme proteins that can be utilized, in principle, for the covalent binding include amino $–NH_2$ (lysine), carboxylic acid –COOH (aspartic, glutamic) and hydroxyl –OH (serine, tyrosine) and cysteine groups. These reactive functional groups on

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the proteins, when targeted for covalent bonding attachment to solids, should be nonessential for the catalytic activity of the enzymes [7].

The characteristics of solid supports that are desirable for biomolecular attachment include large surface area, good chemical, mechanical and thermal stability, hydrophilicity and insolubility. Nonporous materials possess no diffusion constraints, but have very low surface areas for protein binding. The high surface areas of porous materials provide higher protein loading capacity. If most of the surfaces are internal surfaces, however, inefficient diffusion of solutions and significant pressure drop can present major drawbacks. With porous solids, therefore, pore structures must be engineered for efficient diffusion of solutions and minimal pressure drop [5].

The versatile chemical compositions and physical properties coupled with widely available structural forms of polymers have made them excellent candidates as supports for enzyme immobilization. Natural polymers including polysaccharides (cellulose, cellulose derivatives, dextran and chitonsan) and proteins as well as synthetic polymers, such as polystyrene and polyacrylates, have been studied to immobilize enzymes [8–11]. With most polymers, the major barrier is the lack of highly reactive functional groups on the surfaces for direct covalent bonding. Often, surface modification and reactions are needed to fulfill this particular task.

Recently, we have proposed, an alternative matrix for immobilizing catalysts, a product designed as cellulignin [13], which is obtained from biomass acidic prehydrolysis carried out in a steel reactor lined with titanium metal [14]. Cellulignin has high polymerization degree (35% lignin + 65% cellulose) and due to its physical and chemical properties, such as porosity and surface area, showed compatible affinity to be used as immobilizing matrix for lipases, especially for applications under non-aqueous environment [13]. The specific lipase used was from microbial source (*Candida rugosa*).

Enzyme immobilization is often accompanied by changes in enzymatic activity, optimum pH, and affinity to substrate, among others. The extent of these changes depends on the enzyme and carrier support and on the immobilization conditions. Therefore, the current focus of this work is to access the influence of different approaches to activate cellulignin surfaces for optimizing the formation of solid-supported catalysts and compared the biochemical and kinetics properties of the obtained immobilized derivatives in relation to the free enzyme.

2. Materials and methods

2.1. Materials

Commercial *C. rugosa* lipase (Type VII) and *p*-nitrophenyl palmitate (*p*-NPP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The lipase was supplied in lyophilized form with a declared activity of 974 U mg^{-1} solid using Sigma emulsified olive oil as substrate. This lipase is substantially free of protease, and contains lactose as an extender. Polyethylene glycol-1500 (Reagen, SP, Brazil) was used as stabilizing agent of the enzyme. Gum arabic and Triton X-100 came from Reagen (SP, Brazil). Glutaraldehyde (25% solution), carbonyldiimidazole and dimethyl sulfoxide (DMSO) were from Aldrich Chemical Co. (Milwaukee, WI, USA) and sodium metaperiodate was from Nuclear (São Paulo, SP, Brazil). Solvents such as hexane, heptane and 2-propanol were purchased from Synth (São Paulo, SP, Brazil). Heptane was dried with metallic sodium and used as solvent for all experiments. Substrates for esterification reactions (*n*-butanol and butyric acid came from Merck) and were dehydrated, with 0.32 cm molecular sieves (aluminum sodium silicate, type 13 X-BHD Chemicals, Toronto, Canada), previously activated in an oven at 350 °C for 6 h.

2.2. Support

Wood cellulignin from *Eucalyptus grandis* with 3.5% (w/w) moisture content was kindly supplied by RM Materiais Refratários Ltda. (São Paulo, Brazil) in the form of a dark brown powder, having the following properties: porous structure, 35% of lignin; 65% of cellulose, medium particle diameter 1.161 mm; density of 0.35 g cm^{-3}. Due to its acid characteristic, a limiting factor to be used as a support for immobilizing lipases [15], cellulignin was initially neutralized according to procedure described by Gomes et al. [13].

2.3. Support activation procedures

Cellulignin activation with glutaraldehyde was based on the methodology described by de Castro et al. [16]. Initially, the support was submitted to vacuum for 10 min. Under vacuum, buffered glutaraldehyde solution (2.5%, v/v, 0.1 M, phosphate buffer pH 8.0) was slowly added in order to reach a complete solid immersion. Then, the material was transferred to a 100 mL beaker and 4.6 mL of glutaraldehyde solution (2.5%, v/v) was added. The reaction was carried out at room temperature for 1 h. The activated support was filtered and washed with distilled water to eliminate excess glutaraldehyde.

The methodology described by Carneiro-da-Cunha et al. [10] was used for cellulignin activation with carbonyldiimidazole and sodium metaperiodate. Support sample (4 g, dry wt) was immersed in a solution containing carbonyldiimidazole in DMSO (20 mg mL^{-1}) in closed flasks for 2 h at room temperature. Afterward, the support was thoroughly washed with a water:DMSO (1:1) solution and then with water to eliminate excess carbonyldiimidazole. While, for cellulignin activation with sodium metaperiodate, the support sample was immersed in a sodium metaperiodate solution 0.5 M under agitation for 1.5 h in dark place. Later, the active support was transfer to Buchner funnel and washed with distilled water until neutral pH.

2.4. Immobilization procedure

Lipase was immobilized by covalent binding in cellulignin activated with glutaraldehyde, carbonyldiimidazole and metaperiodate in the presence of polyethylene glycol (PEG, MM 1500) as stabilizing agent [13]. Cellulignin samples (4 g, dry wt) were previously soaked in hexane under agitation (100 rpm) for 1 h. The excess solvent was discharged, and the amount of enzyme