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Synthetic *p*-tetrasulphonatocalix[4]arene as novel excipient for lipase-complex

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ABSTRACT

The present article describes formation of excipient-CRL complex from water soluble calix[4]arene derivative (**3** as excipient) and *Candida rugosa* lipase (CRL), which is proposed as a reusable form of enzyme that is free from steric and diffusion limitations associated with those enzymes immobilized onto porous solid supports. The excipient-CRL could completely hydrolyze 50 mM *p*-nitrophenyl palmitate (*p*-NPP) in Tris–HCl buffer at a wide range of temperatures, i.e. 30–80 °C. It is stable under stirred conditions and could be reused multiple times without loss of enzyme activity. It was observed that excipient-CRL complex shows a significant effect on the enzyme activity with an enhancement in thermal stability, while pH and temperature affect the activity of excipient-CRL as well as free CRL. Consequently, the excipient-CRL was found more active than free CRL for the hydrolysis of *p*-NPP in respect of its reusability.

1. Introduction

Lipases (glycerol ester hydrolases, EC 3.1.1.3) catalyze hydrolysis/synthesis of a wide range of soluble or insoluble carboxylic acids, esters and amides. As a tool for organic synthesis, more and more attention has been paid to the lipase-catalyzed reactions in non-aqueous media [1]. Esterification and transesterification are two typical reactions catalyzed by this enzyme [2,3] and numerous important products such as flavor esters [4], monoacylglycerols, optically pure building blocks [5] and biodiesel [6] could be produced by those reactions. Moreover, lipase catalyzed reactions are used in industry for a variety of purposes such as hydrolysis of oils and fats, synthesis of fatty acid esters as cosmetics ingredients or surfactants and production of intermediates for organic synthesis [4–8].

In modern *era* immobilized enzymes play significant role in number of applications like food technology, biotechnology, biomedicine and analytical chemistry [9,10]. Because, the immobilized enzymes have attained a variety of preferences over free enzymes including easy separation of the reactants, products and reaction media; easy recovery of the enzyme, repeated or continuous reuse in order to increase the productivity [11]. The common methods used for immobilization of enzymes are entrapment [12,13] and adsorption [14]. However, another strategy may be the use of excipients [15] for complex formation with enzymes through ionic binding and covalent binding, etc. [16,17]. The excipients-enzyme complex formation may have various preferences over the others, i.e. effective in retaining the enzyme and can achieve high activity with thermal stability.

Excipients generally have well-defined functions in a drug product. As with active ingredients, they may be small molecules or complex and may vary in terms of degree of characterization. They may be chemically synthesized or may be either natural source or biotechnology-derived (recombinant). In contrast to active ingredients, minor components of an excipient may have significant impact on its pharmaceutical performance [18].

Currently, a large number of organic and inorganic materials synthetic polymers and macromolecules have been used as support materials [19,20]. For enzyme immobilization it is very important to choose supports from different nature, which can be classified into three general types: (i) inorganic particles; (ii) synthetic polymers and (iii) natural macromolecules. Nevertheless, synthetic macromolecules such as calixarenes are enormously used in vast fields due to high percentage yield through a single pot synthesis from easily available cheap raw materials. The increasing interest in these compounds and other materials is encouraged by a variety of uses, i.e. medicines, analytical chemistry, nanofibers, nanochips, etc. [21,22]. Recently, calixarenes have been prepared very conveniently and used as a support for enzyme immobilization through covalent linkages [23–26]. However, to achieve the desired goal,

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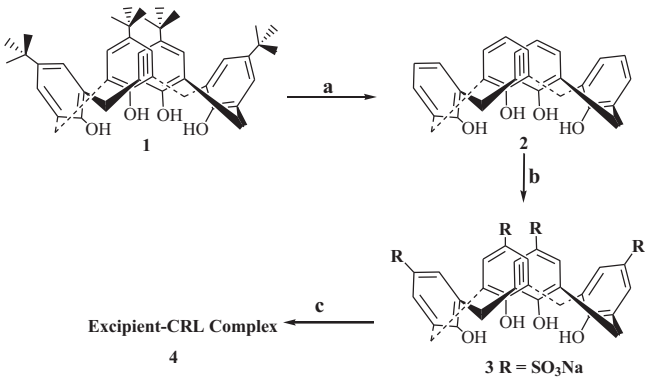

Scheme 1. Schematic synthetic root for the excipient-CRL complex formation of CRL with **3**; (a) $AlCl_3$/phenol/toluene; (b) fuming H_2SO_4; (c) CRL/Tris–HCl buffer).

we have changed the strategy and report herein the immobilization through excipient-CRL complex formation of *Candida rugosa* lipase (CRL) with the water soluble derivative of calix[4]arene, i.e. Na-salt of *p*-tetrasulphonatocalix[4]arene (**3**) to attain the greater activity, significant efficiency at higher temperature and reusability of the enzyme.

2. Experimental

2.1. Materials and methods

Candida rugosa lipase (EC 3.1.1.3), a commercial enzyme obtained from Sigma-Chemical Co. (St. Louis, MO) was used for the excipient-CRL complex formation, Triton-X100 reagent, Bovine Serum Albumin (BSA) and *p*-nitrophenyl palmitate (*p*-NPP) was also purchased from Sigma-Chemical Co. (St. Louis, MO). Other reagents purchased from Fluka, Merck or Aldrich was of standard analytical grade and used without further purification. All commercial grade solvents were distilled and stored over molecular sieves. The aqueous solutions were prepared with deionized water that had been passed through a Millipore milli Q Plus water purification system. UV–vis spectra were recorded on a Perkin Elmer Lambda 35 UV–vis spectrophotometer using standard 1.00 cm quartz cells. The pH measurements were made with pH meter (781-pH/Ion meter, Metrohm) with glass electrode and internal reference electrode. Analytical TLC was performed using Merck prepared plates (silica gel 60 F254 on aluminum). Incubation was performed in a GFL-3032 Incubator. A centrifuge of WIROWKA Laboratoryjna type WE-1, nr-6933 (speed range 0–6000 rpm with a timer 0–60 min, 220/50 Hz, Mechanika Phecyzyjna, Poland) was used for centrifugation.

2.2. Synthesis

p-*Tert*-butylcalix[4]arene (**1**), calix[4]arene (**2**) and Na-salt of *p*-tetrasulphonato-calix[4]arene (**3**) were synthesized according to the previously published procedures [27–29]. The entrapment of CRL with **3** as illustrated in Scheme 1.

2.3. Formation of excipient-CRL complex

2.3.1. Enzyme solution preparation

500 mg of commercial CRL was dissolved in 100 mL of Tris–HCl buffer (0.05 M, pH 7.0). And then the solution used for study was centrifuged at 6000 rpm at 5 °C for 15 min.

2.3.2. Optimization of temperature and pH

The effect of temperature and pH on the reactivity of excipient-CRL was determined by changing the water bath temperature from 30 to 80 °C and pH from 4 to 10; while keeping the substrate (*p*-NPP, *p*-nitrophenyl palmitate) concentration constant (0.5%, w/v).

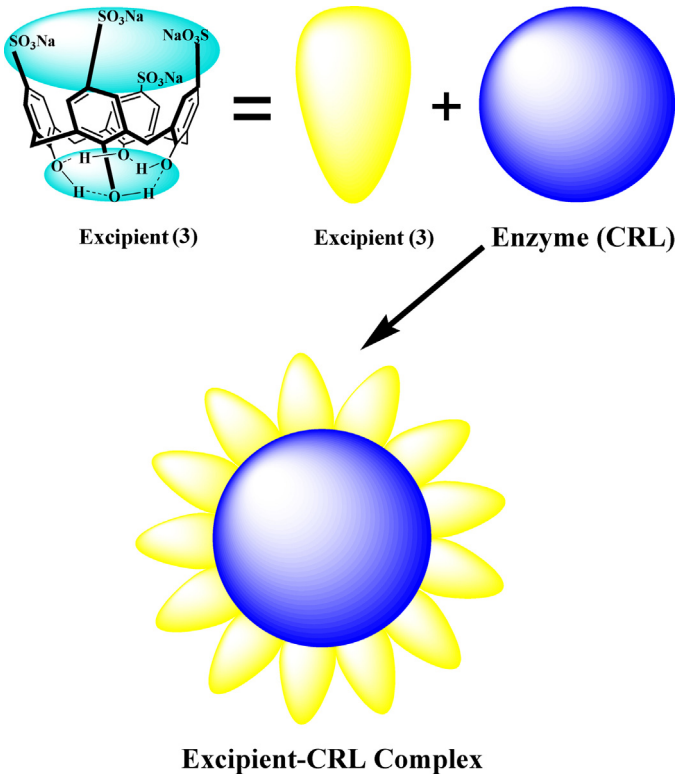

Fig. 1. Proposed schematic representation of CRL in excipient complex formation with **3**. *Conditions*: **3** was used for excipients-CRL complex formation. Each experiment was carried out in Tris–HCl buffer 20 mL (0.05 M, pH 7.0) with 50 μL of enzyme (5 mg/mL); temperature maintained at 25 °C for 4 h and stored for 24 h at 4 °C.

2.3.3. Optimization of time, CRL and support material concentration

By treating various concentrations of CRL with varying amounts of support material at different time intervals for excipient-CRL complex formation showed that the optimum concentration of free CRL should be 50 μL from the prepared CRL (5 mg/mL) solution with 0.2 g of support material (**3**) stirred for 4 h.

2.3.4. Excipient complex formation procedure

For the excipient complex (excipient-CRL) formation of lipase, 0.2 g of **3** were dissolved in 20 mL of Tris–HCl buffer (0.05 M, pH 7), then 50 μL (5 mg/mL) enzyme solution was added in it. After that it was left for 4 h in shaking water bath at 25 °C. Excipient-CRL was stored at 4 °C for 24 h for further treatment. Ethanol was added to precipitate the excipient-CRL. Filtered the solution to separate it from buffer and ethanol; finally washed it with *n*-hexane to remove the lipophilic impurities associated with free enzyme. The use of *n*-hexane makes the entrapped enzyme free from all lipophilic impurities in order to get pure excipient-CRL. The excipient-CRL complex formation was confirmed by taking its TLC (acetone:*n*-hexane in 1:4 ratio) and compared it with the free support, i.e. **3**. It has been observed that free support has greater retardation factor (rf value) as compared to the excipient-CRL. Excipient-CRL was analyzed for expression of bound CRL activity. As the enzyme is a large portentous molecule than calixarene therefore, here it is obvious from the proposed structure that as many calixarene molecules are required to entrap or to form excipient of a single enzyme molecule (Fig. 1). The amount of protein with the excipient (**3**) was determined by using Coomassie Brilliant Blue reagent, following Bradford's method [30], where in the initial and final