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LIPASES: APPLICATION IN CAROTENOIDS ANALYSIS

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Abstract. Enzymes are macromolecules which have the capacity to effect the reactions. Betwwen them, lipases are enzymes that catalyze the hydrolysis of fat, transesterification and esterification among other reactions. Lipase is a versatile enzyme with many potential industrial applications; it has been used for the modification of fats and oils and the synthesis of flavor esters and food additives. The use of immobilized lipase is a possible solution to the high cost of a lipase, because the enzyme can be recovered from the product and reused. Among the various immobilization methods available, immobilization using covalent bonding has been most widely studied. Application on carotenoid extraction from plants can be applied with good yields.

INTRODUCTION

Enzymes are a class of macromolecules with the ability both to bind small molecules and to effect reaction. Without exception, enzymes are proteins and consist of one or more linear chains of, with rare exceptions, the common 20 proteinogenic amino acids. This amino acids with their hydrophilic, hydrophobic, acid and basic side chains are enough both to establish specific binding of substrates and regulator molecules, and to realize the catalytic reaction.

Enzymes, which catalyze a variety of reactions and play an indispensable role in living systems, have many specific features, such as highly controlled regio-, stereo- and substrate-specificity. In addition, enzymatic reactions generally proceed under very mild conditions. Therefore, enzymes have found wide application in the pharmaceutical sciences, chemical synthesis and biotechnology, etc. Even though a large number of efficient chemical reactions have been well developed, enzymes still play a significant role in providing many useful chemical products.

Enzymes are considered as nature's catalysts. Most enzymes today (and probably nearly all in the future) are produced by the fermentation of biobased materials [Louwrier, 1998]. Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds. Lipolytic enzymes are involved in the breakdown of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another [Beisson et al., 2000]. Microorganisms have earlier been found to produce emulsifying agents or biosurfactants to help solubilize lipids [Van Dyke et al. 1991]. Several thousand enzymes possessing different substrate specificities are known, however only comparatively few enzymes have been isolated in a pure form and crystallized, and little has been known about their structure and function.

Until today, more than 3000 different enzymes have been described, but hardly one tenth of them are commercially available.

Lipases (triacylglycerol acyl ester hydrolyses, EC 3.1.1.3) are a family of enzymes that catalyze the hydrolysis of fats (their natural function), transesterification, alcoholysis and

esterification among other reactions. Due to their high activity, selectivity, and the moderate conditions in which they operate, lipases have wide application in several processes usually found in the food, pulp and paper, textile, and leather industries. The high selectivity of lipases is a key feature of these biocatalysts which are used in the resolution of racemic mixtures for preparation of optically pure compounds for the pharmaceutical and agrochemical industries [Goldberg et al., 1990]. In the present day, fat and oil modification is one of the prime areas in food processing industry that demands novel economic and green technologies. Tailored vegetable oils with nutritionally important structured triacylglycerols and altered physicochemical properties have a big potential in the future. Microbial lipases which are regiospecific and fatty acid specific, are very important and could be exploited for retailoring of vegetable oils. Cheap oils could also be upgraded to synthesize nutritionally important structured triacylglycerols like cocoa butter substitutes, low calories triacylglycerols and oleic acid enriched oils. Lipase mediated modifications are likely to occupy a prominent place in oil industry for tailoring structured lipids since modifications are specific and can be carried out at moderate reaction conditions [Gupta et al., 2003].

Although lipase action was initially considered to be restricted to aqueous media, today lipases are employed not only in traditional water-based systems, but also in non-aqueous systems with dissolved substrates and immobilized enzyme. Some non-conventional media that are currently being used include organic solvents, ionic salts and liquids, supercritic fluids, and solvent-free systems. Lower costs, higher substrate concentration, and greater volumetric production are some of the advantages of solvent-free systems.

Water plays multiple roles on lipase-catalyzed esterifications performed in non-conventional media. It is widely known that water is necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes [Yahya et al., 1998]. On the other hand, in esterification/ hydrolysis reactions it is known that the water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media [Basheer et al., 1995]. Particularly for esterifications, as the water content increases, lower equilibrium conversions are achieved.

Although the proper amount of water for an enzymatic reaction depends on many factors (the selected enzyme, support, solvent, co-solvent, and polarity and quantities of substrates), based on the two effects of water mentioned above, some authors agree on the existence of optimum water contents, generally in the range of 0.2–3% [Rocha et al., 1999; Yadav and Piyush, 2003]. For these reactions the activating effect of water dominates at water contents below the optimum, while at higher water contents the net esterification rates decrease, which may be a result of water acting as a substrate in hydrolysis of the acyl-enzyme intermediate [Iso et al., 2001; Svensson et al., 1994].

Enzyme immobilization

Immobilization of enzymes helps their economic reuse and favors the development of continuous biocatalytic processes [Balcao et al., 1996]. Adsorption is the simplest method for enzyme immobilization and is especially suitable for preparing immobilized enzymes for use in organic solvents due to the less desorption of the adsorbed enzymes in an organic environment. As enzymes generally show a much lower activity in organic solvents than in aqueous solutions, it is necessary to improve their activity during the preparation of the immobilized enzymes. For free enzymes, it has been reported that the enzyme activity could be significantly improved by dissolving them in aqueous solutions containing some polar organic solvents such as 2-propanol and acetone or directly dispersing them in pure organic solvents before lyophilization [Jin Chuan Wu et al., 2007].

In the literature we can found different immobilization procedures; due to the multitude of enzymes, spacers and support only a short insight into mobilization techniques can be given. The techniques are: microencapsulation inside Nylon Beads, entrapment in polyacrylamide, covalent immobilization of enzymes on nonporous glass surfaces, immobilization on controlled-pore glass, covalent immobilization to polyamide, immobilization to polyester [Bisswanger, 20004].

Methods used for the immobilization of enzymes fall into four main categories: physical adsorption onto an inert carrier, inclusion in the lattices of a polymerized gel, cross-linking of the protein with a bifunctional reagent and covalent binding to a reactive insoluble support.

Crude lipase (480 mg) was added into 80 ml phosphate buffer (0.01 M, pH 7.0) and the mixture was stirred for 30 min followed by centrifugation at 5000 rpm for 5 min to collect the supernatant. Into 50 ml of the supernatant was added 500 mg adsorbent and the mixture was shaken (300 rpm) for 1 h at room temperature then put into a refrigerator for 24 h. Then the adsorbent was collected by filtration and the amount of lipase adsorbed was calculated according to the difference of lipase concentrations in the solution before and after the adsorption. Lipase concentration was determined based on the UV absorbance at 280 nm. The collected immobilized enzymes were either directly subjected to lyophilization or added into 50 ml pure organic solvents, shaken at 300 rpm for 2 h at room temperature, collected by filtration and then lyophilized [Jin Chuan Wu et al., 2007].

The activity of the immobilized enzymes was markly increased compared to that of the immobilized enzymes prepared by direct lyophilization after the adsorption. 2-Propanol and acetone were the best two solvents for improving the enzyme performances among the solvents tested. No detectable activity was observed when the immobilized *C. rugosa* lipase from Sigma was directly lyophilized after adsorption on Amberlite XAD-7 but considerable activity was observed when the same enzyme preparation was incubated in 2-propanol before lyophilization.

In another work, lipases from *C. rugosa* (CRL), *P. fuorescens* (PFL) and *Candida antarctica* B (CALB), have been immobilized on chitosan powder previously treated with aqueous glutaraldehyde solutions of different concentration. The optimized experimental conditions found for the synthesis of ethyl oleate catalyzed by the chitosan-immobilized lipases were the following: 20% of water, 45 .C, 150 mg of CA/CHIT. Under these conditions 75% of fatty acid conversion was achieved after 7 h of reaction. Reuse of CA/CHIT (the most active biocatalyst prepared) was also achieved. Up to five 24 h uses were possible until significant activity loss was detected. Considering the high substrate concentration of the solvent free systems, the activity and stability of the catalyst allowed the production of 38 mmol of ester in a total time of reaction of 120 h [Foresti and Ferreira, 2007].

Enzymatic hydrolysis of alkali-unstable carotenoid esters at neutral pH by cholesterol esterase or lipase was mentioned by Britton et al., but this was not an extraction procedure. Aravantinos-Zafiris et al. reported an enzyme preparation for the orange peel maceration, but the pigments were released by hexane extraction. Delgado Vargas and Paredes Lopez reported the use of commercial enzyme for the extraction of carotenoproteins from marigold flowers and quantification was done with the hexane.

Enzymic preparations disintegrate the plant tissues to improve the yields in pigment extraction. Usually a combination of cellulolytic and pectinolytic enzymes accelerates the rate of hydrolysis for achieving complete liquefaction. Cellulase randomly splits cellulose chains into glucose whereas commercial pectinase preparations from *Aspergillus niger* have pectinesterase (PE), polygalacturonase (PG) and pectin lyase (PL) activity [Grohman and Baldwin, 1992].

Three different materials, navel orange peel, sweet potato and carrot, were used as good sources of carotenoid pigments. To assess the effects of cellulase and pectinase concentrations and time on yield and color during extraction, different amounts of enzymes were applied to the orange peel, sweet potato and carrot samples. For the orange peel samples, the highest yield was achieved using 5 ml/100 g wet peel pectinase and 0.1 g/100 g cellulose with 12 h extraction. Sweet potato samples showed a similar pattern and maximum yield was reached with the combination of 10 ml/100 g pectinase and 1 g/100 g cellulase after 18 h extraction. The use of 10 ml/100 g pectinase and 0.5 g/100 g cellulase gave the highest yield after 24 h of extraction [Çinar, 2005].

Zorn et al., 2003 studied the enzymatic hydrolysis of marigold flowers and red paprika using two different lipases: commercial lipases and *Plueurotus sapidus* extracellular lipase.

Enzymatic hydrolysis of carotenoid esters could be done using lipases or the extraction of carotenoids from different plant tissues can be more accurate using different types of enzymes in the first step of extraction.

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