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Studies on the specificity of *Penicillium simplicissimum* lipase catalyzed esterification reactions in microemulsions

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Abstract

The enzymatic activity of a new lipase obtained from a *Penicillium simplicissimum* strain has been investigated in esterification reactions of various aliphatic alcohols with natural fatty acids. The reactions were carried out in microemulsions formed in isooctane by bis(2ethylhexyl)sulfosuccinate sodium salt (AOT). The optima pH, T and water content (R) for the enzyme activity in this type of microemulsions have been determined. Studies on the effect of the structure of the alcohols and the chain length of the fatty acids on the lipase specificity have shown a preference for the long chain fatty acids and the secondary alcohols.

1. INTRODUCTION

The potential of enzymes as practical catalysts for the transformation of water insoluble substrates in organic media is becoming increasingly recognized. Among the enzymes so far investigated, lipases is one of the most advantageous because it is stable, inexpensive and widely used in the development of various applications in the detergents, oils and fats, dairy and pharmaceutical industries [1]. In addition to hydrolysis of triglycerides to glycerol and free fatty acids, lipases can be used in esterification and transesterification reactions in low-water content media [2]. This catalytic process is heterogeneous and can be favoured by the use of microemulsion. When these reactions are performed in microemulsion systems, the substrates and products are solubilized in the continuous oil phase, the enzyme is hosted in the water core avoiding direct contact with the organic medium and the reaction can be engineered by changing the water content of the microdroplet, the type of surfactant as well as the composition of the organic solvent used [3,4].

In this work we have studied the various parameters which control the activity of *Penicillium simplicissimum* lipase in microemulsions stabilized by the anionic double tailed surfactant AOT, in isooctane, for the esterification of various alcohols with fatty acids. Also,

he specificity of this lipase is investigated in relation to the structural differences of the ;ubstrates. The *P. simplicissimum* lipase is an extracellular enzyme which shows a high ;tability in water-immiscible organic solvents and it is nonspecific in the hydrolysis of mono-, li- and triolein [5].

?. MATERIALS AND METHODS

?.1. Materials

Lipase from *Penicillium simplicissimum* was purified and characterized according to [5]. The enzyme preparation used in the various experiments had a 99% purity and specific ictivity 142 Units/mg of protein. Bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT) 99% pure vas purchased from Sigma (USA). All alcohols, organic solvents, fatty acids and assay 'eagents used were of the highest commercially available purity. Deionized water was used hroughout this study.

?.2. Preparation of microemulsions

Microemulsions were prepared by adding the appropriate quantities of alcohol and fatty icid, in an isooctane solution containing 100 mM AOT. In this mixture lipase in 50 mM icetate buffer pH=5.0, was added in appropriate amounts to give a final enzyme concenration of 7 units/ml. The final water content depending on the R value, was adjusted by the iddition of the required amount of buffer. A clear solution was obtained after a gentle ;haking for a few seconds. It was assumed that the consumption of the substrates during he reaction did not alter the microemulsion system. A Metrohm Karl- Fischer titrator was ised for water content measurements of AOT in isooctane solution.

?.3. Activity measurements

The reaction was carried out in capped vials placed in a thermostated bath at 35°C. Aliquots of the reaction mixture were withdrawn at selected time intervals and assayed for atty acid content. The rate of esterification was determined spectrophotometrically, by a procedure based on a modification of the Lowry and Tinsley [6] assay in similar fashion to hat performed by Han and Rhee [7]. The depletion of fatty acid was monitored as follows:).1 ml were added to screw cap test tubes containing 4.7 ml of isooctane, 0.2 ml chloroform ind 1 ml of cupric acetate-pyridine (5% w/v, pH=6.0). After centrifugation at 2,500 rpm for I min the free fatty acids were determined in the upper organic phase. The absorbance was ollowed at 715 nm. The slopes of the curves were used to express the enzyme activity.

?.4. Identification of products

The produced esters were isolated by preparative TLC on silica plates (type 60, Merck) ising a solvent mixture of hexane/diethylether/acetic acid (80/20/1). Identification of the products was carried out by IR and NMR spectroscopy.

3. RESULTS AND DISCUSSION

3.1. Reaction conditions

The optimum pH value for *P. simplicissimum* lipase catalyzed esterification reactions in microemulsions was found to be 5.0. This study was performed using as typical reaction the esterification of cyclohexanol with oleic acid. Different enzyme stock solutions with pH values ranging from 3.0 to 8.5 were used (50mM citrate buffer for pH 3-4; 50mM acetate buffer for pH 4-6; 50mM Mes for 6-7; and 50mM phosphate buffer for 7-8.5).

The optimum water content of the microemulsions expressed in terms of the hydration ratio R= $[H_2O]/[AOT]$ was studied in two different reactions of the esterification of hexanol and cyclohexanol with oleic acid and it was found to be 6. Above this R value a constant decrease of the enzyme activity was observed.

Finally the optimum temperature for this lipase in the esterification of cyclohexanol with oleic acid was found to be about 35°C. The optimum values for these parameters which control the enzyme catalytic action in microemulsions, were used throughout this work.

3.2. Effect of primary, secondary and tertiary alcohols on lipase activity

The effect of the structure of alcohols on *P. simplicissimum* lipase activity in microemulsions was performed following the rate of the esterification of pentanol-1, pentanol-2 and 2-methylbutan-2-ol with oleic acid. As it is shown in Figure 1 the rate of the esterification of the secondary alcohol, pentanol-2, is much higher than the esterification rate of the primary

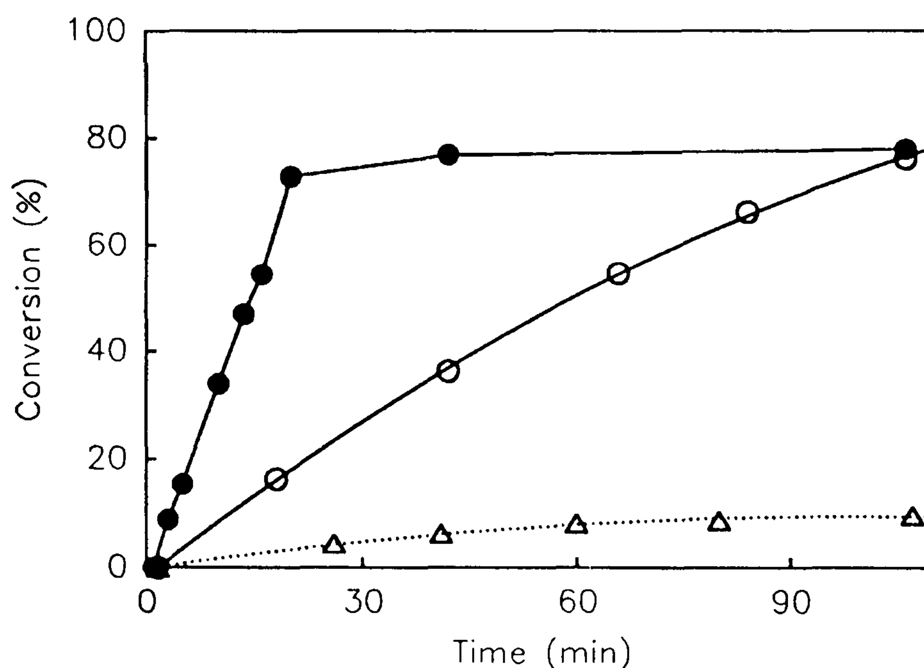

Figure 1. The effect of alcohol structure on the esterification of oleic acid catalyzed by *P. Simplicissimum* lipase in mic nulsions. Degree of conversion as a function of time: (O) pentanol-1; (●) pentanol-2; and (▵) 2-methylbutan-2-ol. Alcohol concentration = 120mM; Oleic acid = 50mM. Assay conditions as described in the text.

pentanol-1, while the tertiary alcohol 2-methylbutan-2-ol was esterified very slowly. The initial velocities were calculated to be 1.850 mM/min for the secondary alcohol, 0.387 mM/min for the primary and 0.067 mM/min for the tertiary alcohol.

This behaviour of *P. Simplicissimum* lipase in these type of reactions can be attributed to the specificity of the enzyme molecule, or to the characteristics of the enzyme containing reverse micellar structure, which may promote the incorporation of the alcohols. Further studies that could clarify this point are actually in progress.

3.3. Effect of alcohol carbon number, n, on lipase activity

The role of the chain length of the alcohol on the lipase activity was examined by studying the esterification reactions of various primary alcohols with different carbon number ranging from n=2 to n=11, with lauric acid. As it can be seen in Figure 2, *P. simplicissimum* lipase shows increasing activity in the esterification of the alcohol with increasing the chain length, up to n=9.

This selectivity can be related to the differences of the partition coefficients of the substrates between the dispersed and the continuous microemulsion phases.

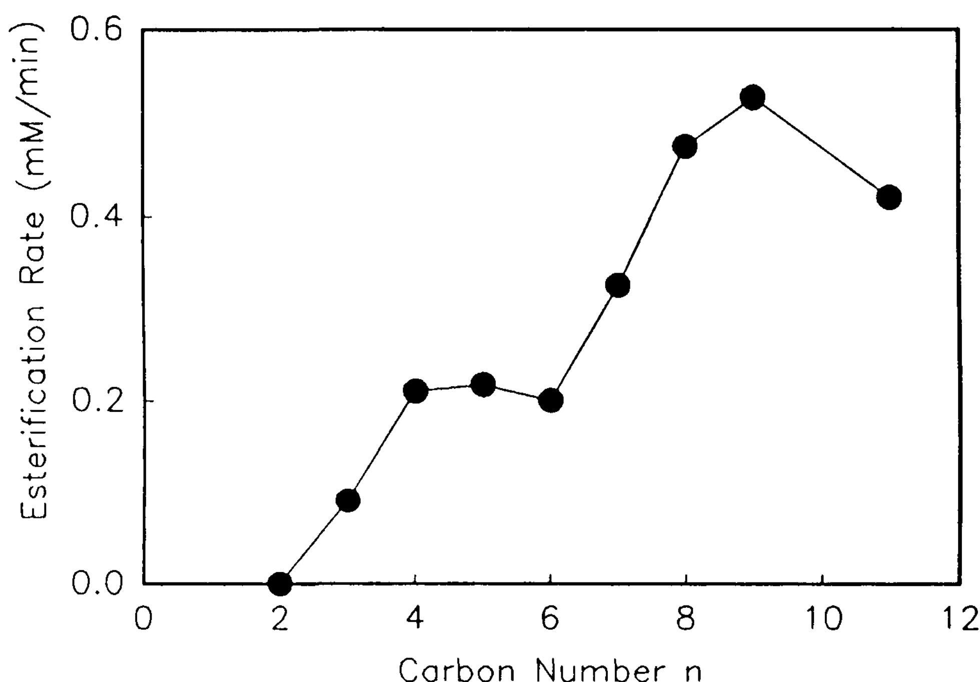

Figure 2. Esterification of oleic acid with various primary aliphatic alcohols by *P. Simplicissimum* lipase in microemulsions. Effect of alcohol carbon number, n, on the initial velocity of the esterification reactions. Alcohol concentration = 150mM; Oleic acid = 50mM. The reaction conditions are described in the Materials and Methods section.

3.4. Effect of fatty acids on lipase activity

The role of the fatty acids in esterification reactions with cyclohexanol catalyzed by *P. simplicissimum* lipase was studied using caprylic, capric, lauric, myristic, palmitic, stearic and oleic acid. Figure 3 shows the degree of conversion of the fatty acids as a function of

time. It seems that the enzyme in this microemulsion system better catalyzes the esterification of oleic, stearic and palmitic acid, with a pronouncedly higher conversion for the latter one.

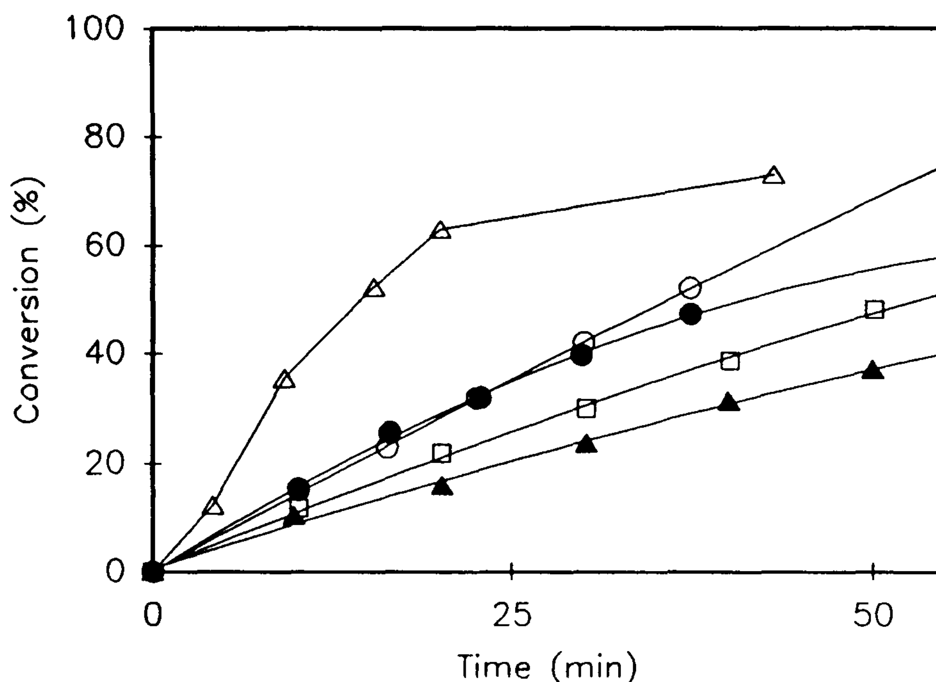

Figure 3. Esterification of cyclohexanol with various chain length fatty acids by *P.Simplicissimum* lipase as a function of time: (□) caprylic; (▲) lauric; (△) palmitic; (●) stearic; and (O) oleic acid. 50 mM of each fatty acid were added in the reaction mixture containing 120 mM of cyclohexanol. All other conditions are as described in the Materials and Methods section.

From this study it can be concluded, that the non-specific, as regards hydrolytic reactions, *P. simplicissimum* lipase, when used in AOT formulated microemulsion systems for the synthesis of esters from alcohols and fatty acids, shows a remarkable specificity for secondary alcohols. Primary alcohols have a lower reaction rate, which relatively increases for the longest ones, while tertiary alcohols present a very slow rate of esterification. This preference is possibly due to the alcohol structure and degree of hydrophobicity, which plays a significant role on its effective concentration in the vicinity of lipase.

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