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IMMOBILIZATION OF LIPASE FROM *FUSARIUM SOLANI* FS1

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SHORT COMMUNICATION

ABSTRACT

Lipase from *Fusarium solani* FS1 was immobilized by covalent attachment to polyacrylamide beads and onto magnetized Dacron, retaining 12% and 97% of activity, respectively. Lipase was also entrapped within polyacrylamide beads, retaining 53% of activity. Investigations of the kinetic characteristics of the immobilized derivatives using triolein as substrate showed that lipase immobilized onto polyacrilamide beads and Dacron did not follow Michaelis-Menten kinetics.

Key words: *Fusarium solani* FS1, immobilized lipase, enzyme kinetics, Dacron, polyacrylamide beads

The use of immobilized lipase facilitates the development of continuous, large scale commercial processes as opposed to the small scale operations which employ soluble enzymes (7). Lipase immobilized on hydrophobic microporous supports yields a higher activity than on hydrophilic supports (8). Immobilization often enhances thermal and chemical lipase stability (it can impart resistance to the denaturing effect of various organic solvents) and leads to predictable decay rates (3).

Immobilized *Mucor miehei* lipase has been used as a biocatalyst in solvent-free esterification which was driven to completion by vacuum distillation of the water produced during the reaction (11).

This work reports the results of covalent immobilization studies of lipases from *Fusarium solani* FS1 using three procedures: i) immobilization onto the outside of polyacrylamide beads via glutaraldehyde; ii) immobilization by entrapment within polyacrylamide beads; iii) immobilization onto the outside of magnetized PET (Dacron), via azide groups.

Lipase used in this study was obtained from the filamentous fungus *Fusarium solani* FS1, isolated from *Carica papaya*. This strain belongs to the Collection of the Phytopathogenic Fungi of Departament of Phytosanity of the University Federal Rural of Pernambuco, Recife, Brazil. The enzyme was precipitated from broth culture with ammonium sulphate at 80% saturation, dialysed against distilled water and lyophilized. Covalent lipase immobilization onto polyacrylamide beads was prepared by placing 60 ml of toluene and 29 ml of chloroform in a sealed Büchner flask with vigorous stirring. Nitrogen gas was fed into the flask through a small tube and 4 ml of distilled water were added. The following reagents were added in sequence: 3.3 ml acrylamide (99%), 5 µl TEMED (N,N,N,N, tetramethyl-ethylenediamine) (99%), 100 µl ammonium persulphate (98%), and 100ml SDS (sodium dodecyl sulphate). The solution was left for 30 minutes until polymerization occurred. Beads were filtered through a nylon filter (150 mesh) and washed with copious amounts of distilled water until the complete elimination of the toluene smell. The beads were stored in distilled water at 4°C.

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The effect of Triton X-100 on the beads was tested adding 0.5g of them to 10 ml of 3.5% Triton X-100 solution (octyl phenoxy polyethoxyethanol) prepared in distilled water. The mixture was incubated for 3 hours at 22ºC. The beads were examined under a microscope and compared to the original beads for evidence of physical damage.

For lipase attachment to the beads, 0.5g of them were mixed with 5 ml of 2.5% glutaraldehyde solution prepared in 0.01 M phosphate buffer pH 7.0 for 30 minutes, followed by exhaustive washing with distilled water. The beads and the lipase solution (10 mg of lyophilized lipase in 5 ml of phosphate buffer pH 7.0) were mixed and stirred for 1 hour at 4°C. The immobilized derivative was washed using the following solutions, in order: 20 ml NaCl (twice), 20 ml tris-HCl 50 mM, pH 8.0 (twice), 20 ml 3.5% Triton X-100 (twice). The immobilized lipase was washed again with the tris-HCl buffer and stored at 4ºC in the same buffer.

Entrapped lipase in polyacrylamide beads was prepared as described above using 30 ml of toluene and l5 ml chloroform. The following were then added in this order: 1.65 ml acrylamide, 2.5 µl TEMED, 50ml ammonium persulphate, 50 µl SDS and 2 ml lyophilized enzyme solution (5 mg/ml) dissolved in water. The solution was stirred for 30 minutes. The beads were filtered, washed in cold distilled water and stored at 4°C in cold tris-HCl buffer 50 mM pH 8.0.

Protein concentration was measured according to Bradford (1).

Lipase activity of the soluble enzyme was initially measured using the pNPP method (10). One unit of enzyme activity was defined as the amount of enzyme that cleaved one µmole of pNPP per minute at pH 8.0 and 25°C. Lipase activity was also determined using triolein as substrate with PVP (1 mg/ml) where the amount of fatty acid produced was measured by the copper method (4). One unit of enzyme activity was defined as the amount of enzyme that catalysed the liberation of one mmole of oleic acid per minute from triolein, at pH 8.0, 25°C.

The activity of immobilized lipase was determined according to the scheme outlined above, except that 8 ml of tris-HCl buffer were added to the test tube and 0.2g of immobilized derivative was added to the emulsion.

In order to convert the magnetized PET-hydrazine groups to azide groups, 0.7g of magnetized PET-hydrazine, obtained according to Leão (6), were suspended in 7 ml 0.6 M HCl, plus 0.8 ml 5% (w/v) $NaN0_3$. The solution was submitted to moderate shaking for 25 minutes at 28°C. The resultant material, PET-azide (Dacron), was separated magnetically and submitted to successive washes with 180 ml distilled water, 180 ml 1 M NaCl, and 180 ml distilled water. For covalent immobilization of lipase onto magnetised Dacron, 0.2g of the Dacron prepared as above were added to 10 ml lipase solution (0.2 mg/ml) and incubated for three hours at 4°C under mild agitation. The immobilized derivative was magnetically separated from the solution and submitted to the following washes: 50 ml NaCl 1 M, 50 ml tris-HCl buffer (pH 8.0, 50 mM), 100 ml Triton X-100 (3.5% w/v), 50 ml tris-HCl buffer, 50 ml NaCl 1 M, 50 ml tris-HCl buffer. The preparation

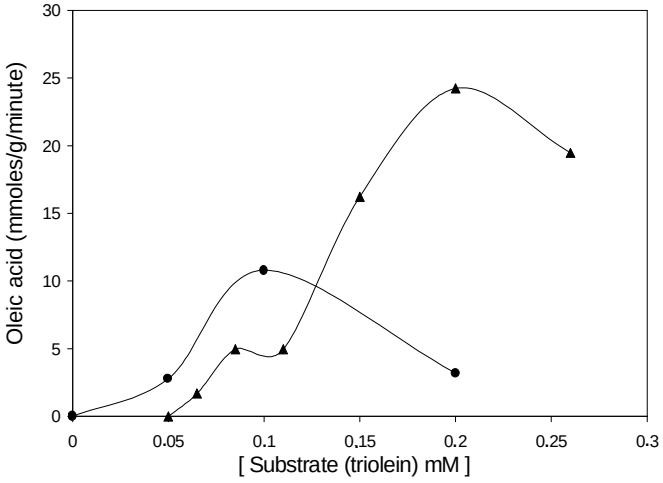

Figure 1. Kinetics of lipase immobilized onto Dacron (●) and entrepped within polyacrylamide beads (▲).

Table 1. Properties of lipase from *Fusarium solani* FS1 immobilized onto polyacrylamide beads and Dacron.

Support	Method used to assay activity	Activity (U/g) support	Activity (U/mg protein)	Activity % soluble enzyme
External polyacrylamide	Colorimetric copper method	6.99	0.80	12.4
External polyacrylamide	pNPP	0.64	7.32x10-2	20.0
Internal polyacrylamide	Colorimetric copper method	12.05	9.42	53.3
Internal polyacrylamide	pNPP	0.75	0.59	47.0
Dacron (Precipitant)	Colorimetric copper method	5.13	2.59	97.0
Dacron (Supernatant)	pNPP	4.33	6.00	71.0

was resuspended in buffer solution and stored at 4°C. The kinetics of the immobilized enzyme was studied using triolein as substrate in concentrations of 50 to 250 mM.

The covalent attachment to polyacrylamide beads via glutaraldehyde resulted in 12% retention of original (soluble) enzyme activity, while covalent attachment to magnetized PET (Dacron) via azide link resulted in 97% retention. Entrapment within polyacrylamide beads resulted in 53% activity retention (Table 1).

The total amount of lipase immobilized within polyacrylamide beads was 8.7 mg of protein per g of support. In comparison, the amount of lipase immobilized onto Dacron was 9.9 mg of protein per g of support. Shaw *et al.* (9) found that the amount of lipase immobilized onto varying supports was dependent upon the length of the linker's carbon chain. The amount of protein immobilized using a linker of two carbons was considerably lower than with a linker of six to twelve carbons (9). When comparing the two linkers used here (glutaraldehyde and azide), the following analogy can be made: glutaraldehyde is comparable to an arm, whilst azide is comparable to a finger. Upon first glance, it may therefore appear that the shorter linker is more effective than the longer linker for lipase binding.

The activities of the immobilized derivatives varied in two ways (Table 1). The lipase activity was found to depend both on the type of immobilization and on the substrate (Table 1). This is in agreement with Lavayre and Barratti (5), who also found that the activity of immobilized enzyme lipases was very dependent on the substrate.

The low activity of immobilized derivatives was found to be due to the difficulty of oil droplets to penetrate the pores of the supports (9). The change in enzyme activity can be due to structural changes caused by the immobilization (2). The differences in activity of the immobilized derivatives according to the substrate (pNPP or triolein) can be seen in the Table 1. The attachment of an enzyme to a support can change the protein conformation and therefore alter enzyme activity. Activity towards larger substrates will therefore be lower as the enzyme cannot change shape to allow the substrate to fit the active site. Clark (2) also states that the microenvironment provided by the support matrix often differs widely from the enzymes natural environment. This can cause abnormal protein conformations, resulting in lowered enzyme activity.

The kinetics of lipase immobilized onto polyacrylamide beads and Dacron can be seen in Fig. 1. It is quite clear that the immobilized lipase no longer follows Michaelis-Menten kinetics. Lipase entrapped within polyacrylamide beads and Dacron presented an inhibition by substrate excess. Most studies have found that one or more properties of immobilized enzymes are different from their soluble form, which was also observed in this work.

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RESUMO

Imobilização de lipase de *Fusarium solani* FS1

Lipase de *Fusarium solani* FS1 foi imobilizada por ligação covalente usando esferas de poliacrilamida e Dacron magnetizado, retendo 12%, e 97% de atividade, respectivamente. A lipase foi também enclausurada em esferas de poliacrilamida e reteve 53% de sua atividade específica. Investigações sobre o comportamento cinético usando trioleína como substrato mostraram que as lipases imobilizadas não seguem a cinética de Michaelis-Menten.

Palavras-chave: *Fusarium solani* FS1, lipase imobilizada, cinética enzimática, poliacrilamida, Dacron

REFERENCES

1. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein – dye binding. *Anal. Biochem.* 72: 248-254, 1976.
2. Clark, D. S. Can immobilization be exploited to modify enzyme activity?. *Trends in Biotechnol.*, 12: 439-443, 1994.
3. Khmelnitsky, Y. L.; Levashov, A. V.; Klyachko, N. L.; Martinek, K. Engineering biocatalytic systems in organic media with low water content. *Enzyme Microb. Technol.*, 10: 710-718, 1988.
4. Kwon, D. Y.; Rhee, J. S. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc.* 63: 89-92, 1986.
5. Lavayre, J. and Baratti, J. Preparation and properties of immobilized lipases. *Biotechnol. Bioeng.* 14: 1007-1013, 1982.
6. Leão, A. B. F. *Imobilização covalente de lipases em polietilenotereftalato (Dacron) magnetizado*. Masters research thesis. Universidade Federal de Pernambuco, 1996.
7. Malcata, F. X.; Reyes, H. R.; Garcia, S. G.; Hill, C. G.; Amundson, C. H. Immobilized lipase reactors for modification of fats and oils – a review. *J. Am. Oil Chem. Soc.*, 67: 890- 910, 1990.
8. Otero, C.; Pastor, E.; Fernandez, V. M.; Ballestero, A. Influence of the support on the reaction course of tributyrin hydrolysis catalysed by soluble and immobilized lipase. *Appl. Biochem. Biotechnol.*, 23: 237-247, 1990.
9. Shaw, J. F.; Chang, R. C.; Wang, F. F., and Wang, Y. J. – Lipolytic activities of a lipase immobilized on six selected supporting materials. *Biotechnol. Bioeng.* 17: 132-137.
10. Winkler, U. K.; Stuckmann, M. Glycogen hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *J. Bacteriol.* 128: 663-670, 1979.
11. Woolley, P.; Petersen, S. B. Lipases: Their structure, Biochemistry and Application. Cambridge University Press, G. B., 1994.