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IN-SITU CROSSLINKING OF *ASPERGILLUS FLAVUS* LIPASE: IMPROVEMENT OF ACTIVITY, STABILITY AND PROPERTIES.

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SUMMARY. The mycelium-bound lipase of *Aspergillus flavus* was stabilised by cross-linkage using glutaraldehyde or methylglyoxal. Lipase activity was enhanced by up to 48% by treatment with methylglyoxal but not by glutaraldehyde. Cross-linking with methylglyoxal increased the thermal stability of the lipase by 58% at 50°C

INTRODUCTION

Mycelium-bound lipase, which may be used as a naturally immobilized enzyme without isolation or purification, has obvious cost attractions in comparison with the use of soluble enzymes in the biotransformation of lipids. However, the commercial value of such a bound lipase relies on its longevity in use, so on both its physical and catalytic activity.

Earlier studies (Long et al. 1996) showed that a Malaysian strain of *Aspergillus flavus* produces a lipase which is bound to the mycelium during growth. A portion of the bound lipase is relatively loosely-linked to the mycelium. After de-fatting the mycelium to remove oil remaining from the culture medium, about 28% of the lipase activity is removed by washing with water. The remaining enzyme is probably bound to the cell wall material by ionic or hydrophobic binding, or both. Thus the binding of the enzyme protein to the cell biomass is weak and impermanent.

To increase the operational stability and minimise the loss of enzyme during use of the bound enzyme, covalent binding of the enzyme to the cellular material using bifunctional materials has been introduced. Glutaraldehyde has been used extensively as a bifunctional cross-linker in immobilizing free enzymes to supports (Kosugi & Suzuki, 1992, Jang *et al.*, 1992, Abdul-Naby,

1993, Carrara & Rubiolo, 1994). However, the *in situ* cross-linking of lipase has been reported less frequently and it has not been attempted previously in *Aspergillus flavus*. This paper describes the effects of using glutaraldehyde and methylglyoxal (acetyl formaldehyde) on the activity and stability of mycelium-bound lipase. In addition the thermal stability of cross-linked mycelium-bound lipase has been compared with untreated material.

MATERIALS AND METHODS

Organism. A. flavus Link (IMI 361648) was grown on a medium containing (w/v) 2% yeast extract, 2% palm olein, 0.3% $(NH_4)_2SO_4$, 0.02% $CaCl_2$, 0.2% KH_2PO_4, 0.1% glucose and 0.4% EDTA-Na. The medium was adjusted to the required pH using 1 M NaOH and sterilised at 121°C for 15 min. Four ml. of spore suspension containing an average of 10^7 spores per ml were used to inoculate 200 ml of the medium. Cultivation was 30°C and 150 rev/min in an orbital shaker.

Lipase preparation a. Mycelium-bound lipase. Mycelium was harvested after 72 h incubation and washed with distilled water, then defatted with 100 ml hexane. The defatted mycelium was used as the source of mycelium-bound lipase. *b. Treated bound lipase.* Four grams of the defatted mycelium-bound lipase was suspended in 150 ml of water containing glutaraldehyde or methylglyoxal at difference concentrations. Cross-linking was allowed to proceed for 30 min at room temperature (25°C). The mycelium was separated by filtration and was washed with 1000ml of distilled water. The effect of the cross-linking on the activity of the mycelium-bound lipase was assayed immediately and compared with the activity of untreated material.

Assay for lipase activity. Lipase activity was measured as described by Sugiura *et al.* (1975) with slight modifications. The reaction mixture consisted of 2.5 ml of 10% refined, bleached and deodorised (RBD) coconut oil emulsified in 10% gum arabic, 2 ml of Tris-HCl buffer pH 8.2, 0.5 ml of 0.25% $CaCl_2$, 4 ml of deionised water and 0.15 g (wet weight) of mycelium (approx. 36 mg dry weight). In the case of free, extracted, lipase, activity was assayed using 1 ml of enzyme solution and 0.25 M $CaCl_2$ was replaced by 0.1 M. The reaction was conducted either at 50°C (for the bound lipase) or 40°C (for the free lipase) for 30 min in a shaker at 200 rev/min and 20 mm displacement. At the end of the reaction, 20 ml of a mixture (1:1 v/v) of acetone and ethanol was added and the amount of free fatty acid liberated was determined by titration to pH 9.0 with 0.05 N aqueous NaOH using an automatic recording pH-stat (VIT 90, Video Titrator, Radiometer, Copenhagen). Activity is quoted as μmol free fatty acids released/min (U).

Extraction conditions for stability study. Four grams of treated or untreated mycelium were suspended in 80 ml of Tris-HCl buffer (pH 8.2, 0.05M) containing 1 mM EDTA. The suspension was shaken in an orbital shaker for 90 min at 35°C and the mycelium was removed by filtration through Whatman No. 4 filter paper. The activity remaining in the mycelium after the extraction was assayed as described above.

Protein determination. Protein was determined colorimetrically by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Hydrolysis of coconut oil. The reaction mixture consisted of mycelium (0.2 g dry weight), 20 ml 10%(w/v) coconut oil in water-saturated hexane and 0.1 ml water. Hydrolysis was allowed to proceed for one hour at 35°C, shaken at 200 rpm. Acetone:ethanol (1:1 v/v) (10 ml) was then added and the free fatty acids content of the mixture was determined by titration using 0.1M NaOH solution and phenolphthalein as indicator.

RESULTS AND DISCUSSION

Glutaraldehyde concentration.

The activity of mycelium-bound lipase decreased with increasing concentrations of glutaraldehyde (Table 1). The mycelium lost 56% of its initial activity when treated with 4% glutaraldehyde. However, the remaining activity was significantly more stable against extraction by water. The highest activity remaining after extraction was obtained using 0.5% glutaraldehyde. Mild cross-linking with 0.1% glutaraldehyde had no significant stabilising effect.

The decrease in lipase activity after glutaraldehyde treatment could be due to the formation of inter- or intra-molecular crosslinkages which may distort the enzyme structure or inactivate a portion of the enzyme molecules but increase the physical stability and rigidity of the enzyme (Wong & Wong, 1992). The formation of cross-linked multimolecular complexes of enzyme protein and mycelial material will increase the extent of the restriction of the diffusion of substrate to, and of products from, the enzyme. Avrameas and Ternynck (1969) noted that insoluble material is formed during the reaction between protein and glutaraldehyde when high concentrations of glutaraldehyde or prolonged reaction times were used. The decrease in the amount of protein extractable from the mycelium after treatment with the higher concentrations of glutaraldehyde (Table 1) suggests that protein cross-linkage has occurred and, therefore, that it is probable that diffusion restriction is increased.

Methylglyoxal (acetyl formaldehyde) concentration.

The lipase activity of mycelium treated with methylglyoxal, increased by up to 48% compared with untreated control material (Table 2). There is no loss of lipase activity, possibly because interaction between lipase and methylglyoxal does not involve amino acids essential for catalytic activity.

Table 1. Effect of glutaraldehyde on the activity and stability of mycelium-bound lipase.

Glutar-aldehyde %(w/v)	Mycelium-bound lipase				Extracted filtrate	
	After treatment		After extraction		Lipase activity (U/g)	Protein mg/ml
	U/g	% of activity	U/g	% of activity		
Control	67.0	100	24.3	36.3	196.5	1.97
0.1	73.0	109	28.4	38.9	24.4	1.92
0.3	65.9	98.4	30.1	45.6	13.0	1.79
0.4	56.9	85.0	36.4	63.9	0	1.26
0.5	53.0	79.1	38.8	73.2	0	1.14
2.0	34.8	52.0	28.3	81.2	0	0.36
4.0	29.3	43.8	25.3	86.1	0	0.31

Table 2. Effect of methylglyoxal on the activity and stability of mycelium-bound lipase.

Methyl-glyoxal (Molar)	Mycelium-bound lipase				Extracted filtrate	
	After treatment		After extraction		Lipase activity (U/g)	Protein mg/ml
	U/g	% of activity	U/g	% of activity		
Control	102.2	100	34.7	34.0	234.2	1.52
0,025	126.4	123.6	40.0	31.7	196.4	1.49
0.1	142.5	139.4	61.9	43.4	165.0	1.43
0.25	151.2	147.9	67.8	44.8	96.6	1.39
0.5	140.1	137.1	68.0	48.5	90.0	1.29

Methylglyoxal was significantly less effective than glutaraldehyde at stabilising the lipase against extraction from the mycelium. According to Torchilin *et al.*(1983) the degree of enzyme stabilization is dependent on the chain length of the bifunctional reagent used and the use of long spacer arms with cross-linkers has been reported to increase the stability of immobilized enzymes (Torchilin *et al.* 1978, Stark & Holmberg 1989). The results presented in Table 2 show that treatment of mycelium with methylglyoxal does not prevent the extraction of enzyme. This suggests that methylglyoxal has not insolubilized protein in the same manner as glutaraldehyde and therefore has not introduced increased diffusion restrictions to the same extent as glutaraldehyde.

Properties of treated mycelium-bound lipase.

The temperature optima for activity of extracted lipase, untreated mycelium and methylglyoxal-treated mycelium (0.5% glutaraldehyde and 0.25M methylglyoxal) were compared. The bound enzymes, treated and untreated, had

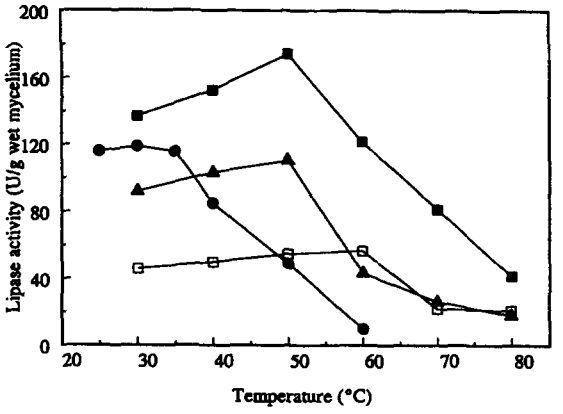

Fig 1 Temperature profile of extracted (free) untreated and treated mycelium-bound lipase.

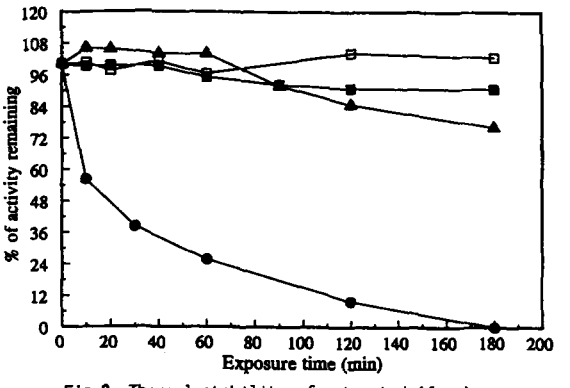

Fig 2 Thermal stability of extracted (free), untreated and treated mycelium-bound lipase.

● extracted lipase ■ 0.25M methylglyoxal ▲ untreated □ 0.5% glutaraldehyde

temperature optima higher than those of the extracted lipase. This implies that they are held rigidly within the membrane structures of the mycelium and consequently are more resistant to thermal inactivation (Fig 1). As reported elsewhere (Kilara *et al.*, 1977, Omar *et al.*, 1988, Shaw *et al.*, 1990) immobilization of lipase almost always leads to positive shifts in the optimum temperature. The optimum temperature for extracted lipase was 30°C (Fig 1) but for untreated and methylglyoxal-treated mycelium the optimum was 50°C. Glutaraldehyde-treated mycelium showed an even higher temperature optimum, 60°C. Methylglyoxal not only increased the stability of bound lipase but also enhanced its activity (Table 2). Compared with untreated mycelium, treatment with methylglyoxal increased the activity of bound lipase by 58% at 50°C (Fig 1). Inactivation of the enzyme at 60°C was greater in untreated mycelium (61%) than in methylglyoxal-treated mycelium (30%). Further, the longevity of lipase in the treated mycelium was greater than that of the untreated mycelium (Fig 2). Glutaraldehyde and methylglyoxal-treated mycelium retained 103% and 91% of their original activity after exposure to 50°C for 180 min. In similar conditions, untreated mycelium-bound lipase lost 26% of its activity and free lipase was totally inactivated (Fig 2). The thermal stability of the untreated bound lipase of *A. flavus* proved greater than that of the lipase from *Penicillium cyclopium* which lost about 66% of its activity on exposure to 50°C for 160 min (Druet *et al.*, 1992).

Batch hydrolysis of coconut oil.

Mycelium treated with glutaraldehyde (0.5%) and with methylglyoxal (0.25M) showed constant activity for 13 cycles of use. In contrast the activity of untreated mycelium fell to that of treated mycelium after 6 cycles of use but

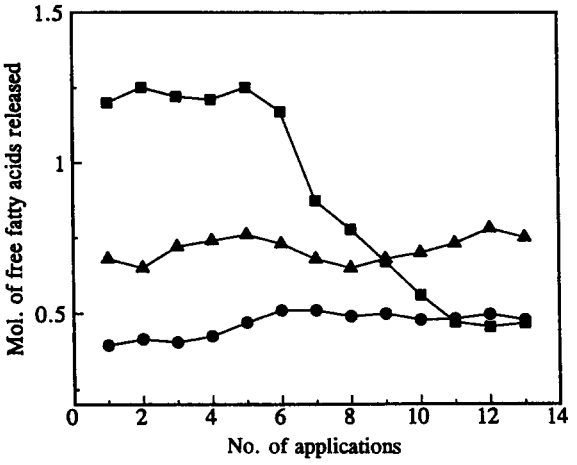

Fig 3 Batch hydrolysis of coconut oil using treated and untreated mycelium-bound lipase.

● 0.5% glutaraldehyde ■ untreated ▲ 0.25M methylglyoxal

then, unexpectedly, after 11 cycles retained a similar activity to that of glutaraldehyde-treated mycelium. In the conditions used, with the medium containing water-saturated hexane, methylglyoxal-treated mycelium did not show the enhanced activity shown in Fig.1.

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