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A chemically modified lipase preparation for catalyzing the transesterification reaction in even highly polar organic solvents

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ABSTRACT

Acylation of *Pseudomonas cepacia* lipase with Pyromellitic dianhydride to modify 72% of total amino groups was carried out. Different organic solvents were screened for precipitation of modified lipase. It was found that 1,2-dimethoxyethane was the best precipitant which precipitated 97% protein and complete activity. PCMC (protein coated microcrystals), CLPCMC (crosslinked protein coated microcrystals), EPROS (enzyme precipitated and rinsed with organic solvents) and pH tuned preparations of modified and unmodified lipase were prepared and used for carrying out transesterification reaction with *n*-octane and dimethyl formamide (DMF) as reaction medium. In *n*-octane, among all the preparations, CLPCMC of modified lipase gave highest rate (1970 nmol min^{-1} mg^{-1}) as compared to unmodified pH tuned lipase (128 nmol min^{-1} mg^{-1}). In DMF, with both 1% (v/v) and 5% (v/v) water content, CLPCMC showed highest initial rate of 0.72 and 7.2 nmol min^{-1} mg^{-1}, respectively. Unmodified pH tuned lipase showed no activity at all in DMF with both 1% and 5% (v/v) water content.

Lipases have found large number of applications in catalyzing transesterification/esterification reactions in nearly anhydrous organic solvents.[1,2] While enzymes, in general and lipases in particular work well for organic synthesis and kinetic resolution in such media,[3,4] two key issues remain. Firstly, the initial rates are not as high as the catalytic efficiency of enzymes in more conventional water rich media.[5–8] Secondly, most of the enzymes show even further poor activity in the presence of polar organic solvents like DMSO or DMF.[8,9] The accepted view is that essential water layer around the enzyme is stripped off by these highly polar solvents.[5,8] This is important for organic chemists as many organic compounds of interest do not have appreciable solubility in highly nonpolar solvents like *n*-octane (a solvent often used to measure initial rates in nonaqueous enzymology[6,8]). Rees et al.[10] modified α-chymotrypsin with pyromellitic dianhydride (PMDA)[11] to confer large number of additional negative charges on the protein surface in an attempt to enable the protein to compete better for water with organic solvents (Fig. 1). The lyophilized powders of the modified enzyme, in fact gave lower rates than even those of unmodified lyophilized powder. In an insightful analysis, the changes in the ionic state during lyophilization was found to be responsible for this unexpected result. A later work from our laboratory showed that the same modification with α-chymotrypsin did indeed result in a biocatalyst preparation which gave high transesterification activity in low water media, provided 'drying' of enzyme (the removal of excess water) is carried out by precipitation rather than lyophilization.[12] Also, in that case, the preparation was found to shift a_w for giving maximum initial rate from 0.97 to 0.33 in acetonitrile.[12] These results with α-chymotrypsin indicated that the additional charges placed on the enzyme as a result of chemical modification did enable the enzyme to hold on to essential water molecules when the enzyme was placed in a reasonably polar and water soluble organic solvent like acetonitrile.[12] In the present work, a two-pronged strategy based upon the following has been attempted (a) modifying the enzyme with PMDA[13,14] (b) drying the enzyme by precipitation with organic solvents with or without the presence of additives and also followed by subsequent chemical crosslinking with glutaraldehyde.[15–21] It is shown that as a result of this strategy *Pseudomonas cepacia* lipase can catalyze transesterification reaction even in highly polar organic solvents like DMF.

TNBS assay[22] showed that 5 out of 7 free lysine amino acids were modified with PMDA. The degree of modification of free amino groups was calculated to be 72%.[23] Different high performance biocatalyst preparations (PCMC[16] CLPCMC[21], EPROS[19] and pH tuned lyophilized powder (lyophilized from a solution in aqueous buffer at pH 7.0, which is the optimum pH for this enzyme in aqueous buffers[5,24])) of modified lipase were prepared and its transesterification activity (1-hexanol with tributyrin)[25–27] was measured with *n*-octane and DMF as reaction media.

Figure 2 shows the activity recovery[28] and amount of protein precipitated[29] when different organic solvents were used as precipitants. 1,2-dimethoxyethane was found to be the best precipitant and hence for the preparation of all high performance

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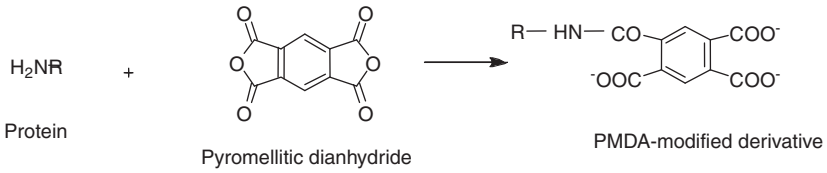

Figure 1. Chemical modification of protein with Pyromellitic dianhydride (PMDA).

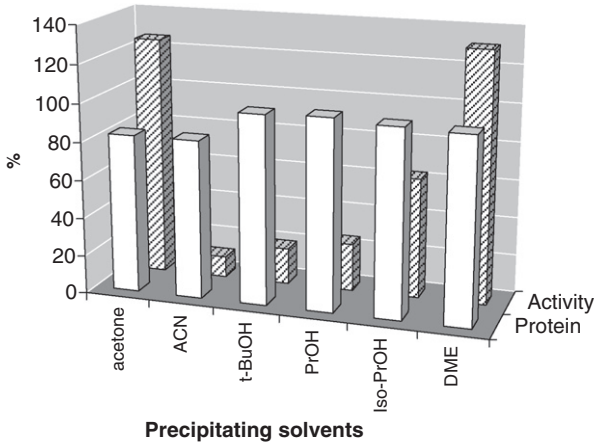

Figure 2. Effect of different precipitating solvents on PMDA modified *P. cepacia* lipase. Lipase was precipitated with different precipitating solvents. Then the lipase hydrolytic activity and protein precipitated were determined after re-dissolving the precipitate in an aqueous buffer.

biocatalyst preparations, 1,2-dimethoxyethane was chosen as the precipitating solvent.

Table 1 summarizes the catalytic performances of pH tuned lyophilized powder, EPROS, PCMC and CLPCMC of unmodified and modified lipase. Much of the early work on enzyme catalysis in nearly anhydrous media was carried out using such pH tuned lyophilized powders. Hence, this preparation has been included as a 'control' to compare with. The initial rates obtained during the assay (Table 1) showed that CLPCMC was the best catalyst followed by PCMC > EPROS > pH tuned in *n*-octane. For all the preparations, biocatalyst formulations of modified lipase were better than corresponding formulation of unmodified lipase. The initial rates showed by CLPCMC of modified lipase (1970 nmol min^{-1} mg^{-1}) were 15.4 times higher than the initial rates showed by pH tuned unmodified lipase (128 nmol min^{-1} mg^{-1}).

After this, these preparations were evaluated for catalyzing the transesterification reaction in DMF with two different water contents (1% (v/v) and 5% (v/v)) (Table 2). Again, the performance of all the three preparations (EPROS, PCMC and CLPCMC) exceeded well beyond the pH tuned lyophilized powders of lipase (which showed no activity at all). Also, the modified lipase preparations were better than unmodified ones. With both, 1% (v/v) and 5% (v/v) water content, CLPCMC of modified lipase performed best among all the preparations. With 1% (v/v) water content in DMF, CLPCMC of modified and unmodified lipase gave initial rates of 0.72 and 0.05 nmol min^{-1} mg^{-1}, respectively (Table 2). At same water content, PCMC and EPROS of both modified and unmodified lipase showed very low catalytic activity. With 5% (v/v) water content, CLPCMC of modified and unmodified lipase gave initial rates of 7.2 and 2.5 nmol min^{-1} mg^{-1}, respectively (Table 2). It is interesting to note that% (v/v) water which was found better (out of 1% and 5%) was different for various enzyme preparations. PCMC of both unmodified lipase and modified lipase worked better with 1% (v/v) water. In all other cases, 5% v/v water gave better catalytic rates. This means that water partitioned away from the organic solvent by the enzyme did not meet its optimum hydration level for maximum activity with 1% (v/v). With 5% (v/v) water content, PCMC of both modified and unmodified lipase showed very less activity (<0.001 nmol min^{-1} mg^{-1}). That is understandable as at 5% (v/v) water, it is likely that salt core would start dissolving. It is interesting to observe that crosslinking in case of CLPCMC seems to have at least partially avoided this problem. The initial rates observed with modified EPROS were 0.42 nmol min^{-1} mg^{-1} and with unmodified EPROS were 0.19 nmol min^{-1} mg^{-1}.

To sum up, this work shows that by using the synergy between enhancing changes in the enzyme surface (by chemical method) and 'drying' (removal of excess water) enzymes by precipitation in the presence of additives and subsequent cross linking yields biocatalyst preparations which work better than lyophilized powders in nearly anhydrous media. In this work, *P. cepacia* lipase has been chosen as this is known to be among the best lipase for catalyzing transesterification reaction.[30] The possibility of using lipases in organic solvent like nearly anhydrous DMF opens up further opportunities for using lipases in organic synthesis and kinetic resolution. Right from early days of nonaqueous enzymology, this has been a much sought after goal. Early efforts using protein

Table 1
Transesterification reaction of tributyrin with *n*-hexanol catalyzed by different biocatalyst formulations of *P. cepacia* lipase with *n*-octane as a reaction medium

Lipase formulation	Initial rates (nmol min^{-1} mg^{-1})	Fold increase
pH tuned (unmodified)	128	1
EPROS of unmodified lipase	240	1.9
PCMC of unmodified lipase	620	4.8
CLPCMC of unmodified lipase	974	7.6
pH tuned (modified)	513	4.0
EPROS of PMDA modified lipase	472	3.7
PCMC of PMDA modified lipase	1150	9.0
CLPCMC of PMDA modified lipase	1970	15.4

Initial rates were calculated from the aliquots taken within 0.25–1 h. The percentage conversions during these time periods were in the range of 1–25% and were in linear range. Enzyme used was biocatalyst formulation made from 2 mg solid enzyme (in 2 ml of reaction volume) in each case.

Table 2
Transesterification reaction catalyzed by different biocatalytic formulations of PMDA modified *P. cepacia* lipase with DMF as a reaction medium

Biocatalyst preparation	Initial rates (nmol min^{-1} mg^{-1})	
	1% (v/v) water	5% (v/v) water
pH tuned	0	0
EPROS of unmodified lipase	<0.001	0.19
EPROS of modified lipase	<0.001	0.42
PCMC of unmodified lipase	0.01	<0.001
PCMC of modified lipase	0.02	<0.001
CLPCMC of unmodified lipase	0.05	2.5
CLPCMC of modified lipase	0.72	7.2

Initial rates were calculated from the aliquots taken within 1–5 h. The percentage conversions during these time periods were in the range of 1–20% and were in linear range. Enzyme used was biocatalyst preparation made from 2 mg solid enzyme (in 2 ml of reaction volume) in each case.

engineering[31] and lately through directed evolution[32] have been made to obtain enzyme mutants which can function in the aqueous cosolvent mixtures containing high percentage of DMF. In that context, CLPCMC, is a simple biocatalyst preparation for any organic chemist to convert and use any commercially available lipases for catalyzing reactions in highly polar organic solvents.

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