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Rhizomucor miehei lipase remains highly active at water activity below 0.0001

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The lipase from *Rhizomucor miehei* adsorbed on polymer beads retains substantial catalytic activity even after exhaustive drying, and the use of dry box procedures to prevent entry of atmospheric water. Rates of esterification and transesterification (alcoholysis) were measured while stirred in hexane pre-dried to similar low water activity (a_w). The rate of dodecyl decanoate synthesis was over 30% of that at the optimum (a_w 0.55) after drying with anhydrous $CuSO_4$ ($a_w<10^{-3}$) or MgO ($a_w<10^{-4}$). Freshly reactivated molecular sieve could cause a further reduction in, but not elimination of, activity.

Lipase (EC 3.1.1.3); Water activity; Organic media; *Rhizomucor miehei*

1. INTRODUCTION

It is now widely recognised that many enzymes retain catalytic activity at low residual water contents. This is particularly easily demonstrated in the presence of an organic liquid phase that permits transfer of the reactants. Quantitative limits may be expressed either as the weight of water bound by the protein, or the thermodynamic water activity (a_w) of the system; these can be related via the adsorption isotherm. Catalytic activity of moist enzyme powders in air is said [1] usually to require at least 0.07 g water/g (a_w=0.2–0.3), while enzyme-catalysed processes have been described at a_w as low as 0.1 in foods [2] and in a solid–gas system [3]. In organic media, enzymic activity has been reported at a_w of about 0.1 [4]; and with an enzyme water content of about 0.02 g/g [5], corresponding to a similar a_w. We recently found that one lipase, from *Rhizomucor miehei* (used in carrier-adsorbed form), still showed high activity in a system pre-equilibrated to a_w of below 0.01 [6]. We now report further work, using strict precautions to prevent entry of water from the environment (never previously thought necessary in studies on enzymes). This has allowed extension of the lower a_w limit by at least 2 orders of magnitude, to less than 0.0001.

2. EXPERIMENTAL

2.1. *Catalysts and reactions*

Two immobilised derivatives of *Rh. miehei* lipase were used, with the supports being an anion exchange resin ('Lipozyme IM 20' preparation, kindly given by Novo-Nordisk AS) and Accurel EP 100 macroporous polypropylene (immobilised by adsorption [7]). Esterification of dodecanol and decanoic acid in hexane was measured as described [6]. Synthesis of octyl decanoate by transesterification from 0.5 M octanol and 0.5 M dodecyl decanoate in hexane was also determined.

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2.2. *Water equilibration*

The catalyst and the organic phase were first pre-dried with molecular sieve as previously described [6]. They were then further equilibrated through the vapour phase in sealed jars: with a new charge of molecular sieve 4A that had been freshly re-activated by heating at 350°C; or with MgO or anhydrous $CuSO_4$. About 20 g of drying agent was used with about 100 mg catalyst or 5 ml organic phase.

2.3. *Prevention of entry of environmental moisture*

This could easily cause significant changes in catalyst hydration and a_w in these exhaustively dried systems. Therefore, removal of the catalyst and organic phase from the jars in which they had been equilibrated, their mixing, and the entire reaction were performed inside a fully-sealed glove box pre-equilibrated overnight with P_2O_5. This is known as rapid drying agent, generally considered very intense, though the resultant a_w may be as high as 10^{-4} (calculated from published data [8]).

2.4. *Estimation of residual water content of catalysts*

Water in solution in dimethyl sulfoxide was determined by coulometric Karl Fischer titration (Metrohm 684KF Coulometer). Syringes used to inject samples through the septum of the titrator were oven dried, rinsed twice with dried solvent, and then with the sample. Dimethyl sulfoxide in a septum-sealed bottle was dried with molecular sieve 4A to a residual water content of 50 ppm. Catalyst (0.6–1.1 g) was pre-equilibrated in a vial which was then sealed with a septum cap, and 3 ml dried dimethyl sulfoxide was added via a dried and rinsed syringe. After extraction for 2–4 days, 0.5 ml portions of the supernatant were transferred to the titration cell.

3. RESULTS AND DISCUSSION

The immobilised enzymes and organic phases were pre-equilibrated through the vapour phase with appro-

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priate drying agents. In our previous studies [6] at higher a_w values, equilibration was complete within 7 and 1 days, respectively. With only traces of water remaining in the current experiments, there was no detectable weight change of the catalysts, and the water content of the organic phase was zero by Karl Fischer analysis. Hence we have had to use long equilibration periods, monitored just by the measured reaction rate.

One drying agent used was a freshly reactivated molecular sieve, which should give a very low a_w value, though one hard to specify, since the drying action is via adsorption. Hence we have also used two drying agents that act by interconversion of defined chemical species: $CuSO_4$ (converting to $CuSO_4 \cdot H_2O$) and MgO (converting to $Mg(OH)_2$). Values for the equilibrium a_w obtained with these pairs are not as certain as for the saturated salt solutions used for equilibration to higher a_w values. Measurements of their efficiency as drying agents are usually partly kinetically determined. Some direct measurements of the very low equilibrium water vapour pressures at ambient temperatures have been reported, but these are technically difficult and vary quite widely. For $CuSO_4$+$CuSO_4 \cdot H_2O$, it has been argued convincingly [9] that a value of 0.017 mmHg at 25°C is correct, corresponding to a_w of 7.2×10^{-4}. An alternative is to calculate the standard free energy for dissociation, and hence a_w, from the tabulated values for formation of both species and of liquid water [10]. This yields a_w values of 4.4×10^{-4} for $CuSO_4$+$CuSO_4 \cdot H_2O$, and 4.1–14×10^{-6} for MgO+$Mg(OH)_2$, depending on the particle size of the former [11].

Table I shows initial reaction rates of immobilised lipases pre-equilibrated for between 90 and 120 days under exhaustive drying conditions. During esterification, the water formed as a reaction product caused a significant acceleration of the observed rate as the reaction proceeds, making estimation of the initial rate harder. Hence we have also determined activity as a catalyst of a transesterification (alcoholysis) reaction, in which water is not stoichiometrically involved. In all cases the rates are not very different from those found with the same catalyst after the initial drying stage. Rates determined after between 7 days and 50 days did not show significantly higher activities. Hence the results shown in Table I seem to relate to at least a metastable state of the enzyme. Drying with molecular sieve does cause a significant fall in the alcoholysis activity, though the contrast with esterification may simply reflect differences in the drying effectiveness of apparently identical batches of molecular sieve.

TABLE I

Initial reaction rates catalysed by exhaustively dried immolised *Rh. michei* lipase

Pre-incubated with:	Wateractivity	Reaction rate (mmol $s^{-1} \cdot kg^{-1}$)			
		Alcoholysis		Esterification	
		AER[a]	PP[a]	AER[a]	PP[a]
Molecular sieve	?	0.10	2.1	0.69	3.7
$MgO/Mg(OH)_2$	1–14×10^{-6}	0.39	2.8	0.76	3.5
$CuSO_4/CuSO_4 \cdot H_2O$	4– 8×10^{-4}	0.32	2.8	0.69	3.7
(Initial drying)	< 0.01	0.10	2.8	0.76	3.9
LiCl sat.	0.12	0.32	3.1	0.83	4.9
$Mg(NO_3)_2$ sat.	0.55	–	–	1.77	10.3

[a] The lipase was adsorbed to either anion exchange resin (AER) or macroporeus polypropylene (PP).

For comparison, Table I also shows the reaction rates after pre-equilibration at a_w values of 0.12 and 0.55 (the optimum for the esterification reaction [6]). As may be seen, the activity of the immobilised lipases on either support after exhaustive drying remains a significant fraction of that at such higher a_w values.

There are three possible types of explanation for the substantial activity retained by this supported lipase even after equilibration at very low a_w. (i) This enzyme may remain active in the complete absence of any water molecules. (ii) Some essential water on the enzyme may be very tightly bound in a thermodynamic sense, as in some salt hydrates like $CuSO_4 \cdot H_2O$. (iii) The rate of desorption of essential water may be extremely slow. In view of the known hysteresis of water binding by proteins, (ii) and (iii) may be difficult to distinguish in practice.

By the most common method of determining water content of proteins, weight compared with extensively dried material, our preparations have zero water content by definition. We have attempted to determine whether some water molecules are still present by extraction with dried dimethyl sulphoxide, which should dissolve the protein and any associated water. Immobilised lipases, exhaustively dried over molecular sieve, gave results corresponding to residual water contents of 0.004 g/g (anion-exchange resin) and 0.0025 g/g (polypropylene). However, the possibility of interference by extracted material or water production by chemical reaction during extraction cannot be ruled out.

Two important practical consequences follow from the ability of this enzyme to remain active at exceptionally low a_w. Firstly, this a_w will lead to a large mass action effect on hydrolytic equilibria, and hence on synthetic yields where water is a product. For example, a relatively unfavourable esterification giving just 0.1% synthesis from 1 M reactants at a_w close to 1 would be shifted to a preparatively useful 73% yield at a_w of 10^{-4}. Secondly, in any esterification reaction exhaustive drying conditions may be applied to remove product water without risk of complete loss of catalyst activity.

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