Factors Governing the Activity of Lyophilised and Immobilised Lipase Preparations in Organic Solvents

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Active site titration and activity measurements were performed in hexane on lyophilised lipase preparations containing different amounts of phosphate buffer and lipase immobilised on porous polypropylene. Lyophilisation of Thermomyces lanuginosus *lipase with large quantities of phosphate salts (200 mM) increased the specific activity fourfold, and the number of rapidly titratable active sites increased to 50% from the 13% observed when smaller amounts of phosphate buffer were used (20 mM) during lyophilisation. The phosphate buffer worked as an immobilisation matrix for the lipase, and the increase in specific activity was at least partly due to decreased mass transfer limitations. When lipase was immobilised on porous polypropylene, the specific activity was 770 times higher than that of the best freeze-dried preparation. At optimal enzyme loading, 93% of the enzyme molecules were titrated at a high rate; this indicates that this adsorption on a hydrophobic surface was a very efficient means of reducing mass transfer limitations and of immobilising the enzyme in its active conformation for use in organic solvents. The variation in specific activity with water activity was found to correlate very well with the variation in titratable active sites when lipases from* Burkholderia cepacia *and* Thermomyces lanuginosus *were immobilised on porous polypropylene. The catalytic activity per competent active site was thus constant over the whole range of water activities.*

KEYWORDS:

enzyme catalysis · immobilisation · lipases · solvent effects · water activities

Introduction

The use of enzymes in organic solvents is now well established, and some companies use biocatalysis in organic solvents as an alternative to classical chemical synthesis. BASF (Germany), for example, has developed large-scale processes for lipase-catalysed resolution of racemic amines and alcohols.[1] The widespread use of enzymes as catalysts in organic solvents has been limited, however, by the low activities obtained for enzymes in organic solvents relative to those in aqueous solutions.[2]

To overcome this limitation, several strategies have been employed. It is believed that an important reason for the low activity is reversible denaturation of the enzyme during the lyophilisation of the enzyme prior to its addition to the organic solvent. Addition of different types of additives—crown ethers,[3, 4] sorbitol,[5, 6] PEG (poly(ethylene glycol))[7] and methyl-β-cyclodextrin[8]—is one approach to the suppression of the inactivation process. Other types of additives that have been employed are different types of salts, such as KCl.[9, 10] The positive effect exhibited by these salts could be due to several mechanisms. They may protect the enzyme from direct inactivation by the solvent or also work as lyoprotectants. It is also possible that the different additives employed in the lyophilisation might serve as immobilisation matrixes for the enzyme and that the increased activity could be partly due to reduced diffusion limitations in the biocatalyst.[11, 12]

Covalent attachment of poly(ethylene glycol) derivatives,[13] lipid/detergent coating of the enzyme,[14, 15] formation of a noncovalent enzyme polymer complex during lyophilisation[16] and the use of reversed micelles[17, 18] are all strategies that have been employed to solubilise enzymes in organic solvents and thereby increase the catalytic activity.

Immobilisation has proved to be an efficient method to improve catalytic activity in organic solvent.[19–21] Immobilised biocatalysts facilitate mass transfer by spreading the enzyme over a larger surface area and by preventing the enzyme particles from aggregating.

Different methods of using lipases in organic solvents, such as immobilisation by adsorption onto Accurel EP-100, entrapment into a sol-gel, detergent coating and freeze-drying in the presence of KCl or crown ether, have recently been compared.[12] Immobilisation by adsorption onto Accurel EP-100 was found to be the method of choice, since high specific activity could be combined with high protein loading and high protein recovery.

Active site titration is a valuable method for elucidation of changes in enzyme activity upon changing the enzyme formulation or reaction conditions. Information can be obtained as to whether any increase or decrease in activity is due to a change in the total amount of active enzyme molecules or due to differences in the catalytic activity per active enzyme molecule.

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Active site titration was therefore performed in order to gain further insight into the mechanism behind the activity obtained for the Accurel EP-100 preparation and different freeze-dried preparations. A previously described method for active site titration[22] of lipases in aqueous solution and in organic solvents was adopted for those lipase preparations. The enzyme was irreversibly inhibited by methyl *p*-nitrophenyl *n*-hexylphosphonate, which reacts specifically with the active site serine residue.[23] The *p*-nitrophenol released upon binding can be measured spectrophotometrically and a quantification of the amount of active enzyme molecules in the preparation can be obtained. Active site titration of lipases from *Burkholderia cepacia* and *Thermomyces lanuginosus* was performed at different water activities in an attempt to elucidate the mechanisms behind the water-activity profiles observed for these two lipases.

Results and Discussion

Active site titration

Active site titration was carried out on different lipase preparations. Lipase molecules adsorbed onto the porous polypropene material EP-100 were effectively titrated within 24 hours (Figure 1). On the other hand, when lyophilised preparations were

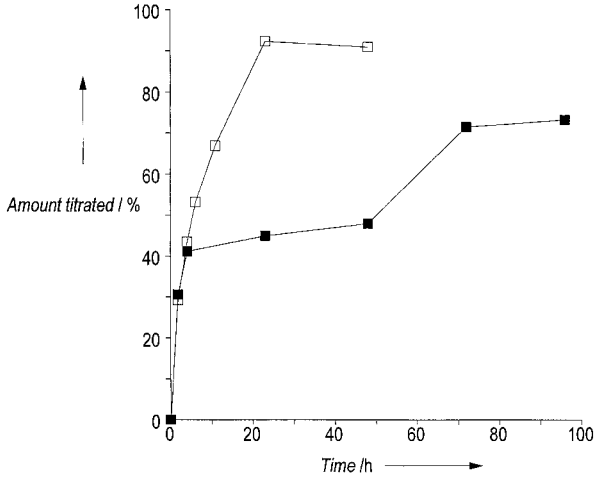

Figure 1. *Progress curves for the amount of titrated enzyme (% of protein content) of* Thermomyces lanuginosus *lipase immobilised onto Accurel EP-100 (□) or freeze-dried in the presence of 200 mM phosphate buffer (■). The protein loading was 55 mg g^{-1} on Accurel EP-100 and 100 mg g^{-1} for the freeze-dried preparation. The active site titrations were performed in hexane/diethyl ether (97:3). The concentration of the inhibitor MNPHP was 0.36 mM. The* p-nitrophenol *released was extracted with 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0) when the lipase was immobilised onto Accurel EP-100 and with 200 mM phosphate buffer (pH 7.0) when the freeze-dried preparation was titrated. The errors in the active site titrations were $< 5\%$.*

titrated, not even 96 hours was enough to come to a distinct end point. In those preparations, a proportion of the active sites was titrated at a rate similar to that obtained with lipase adsorbed onto EP-100, while another proportion of the enzyme molecules titrated at a much lower rate. The rapidly reacting lipase molecules are clearly those that are active and easily accessible for the reagent used in titration. The slowly reacting ones might be buried and therefore subject to mass transfer limitations, or they might be present in a less-active conformation. It is well known that most types of lipase molecules can exist in open (active) and closed (inactive) forms depending on the position of the "lid" region.[24] It might thus be speculated that the enzyme molecules reacting rapidly in the titration would be those present in the open conformation, whilst the slowly reacting ones would thus be those present in the closed conformation. The rate of titration of those molecules might thus represent lower activity of the closed form of the lipase or a slow conversion into the active open form. If this interpretation is true, lipase adsorbed on polypropylene is present almost exclusively in the open form, and this is therefore a good method by which to activate the enzyme. It is well known that many lipases, including those used in this investigation,[25, 26] are activated at interfaces between aqueous and organic phases, and it has also been reported that they can be activated by hydrophobic solid surfaces. It has been speculated that lipase molecules in organic media might be activated by the medium. However no such activation was observed in a careful investigation into the existence of interfacial activation of lipase molecules in organic media.[27]

For the lyophilised preparation, 70% of the active sites were titrated after 96 h of titration (Figure 1). It is possible that the remaining 30% were reversibly denatured during the freeze-drying step[5] or directly denatured by the solvent; this would explain why some proportion of the enzyme molecules are not available for catalysis. Titration times of longer than 24 hours are not suitable, since inactivation of lipase molecules would be expected to interfere with the determination. The active site titrations were therefore fixed at 24 h and the active sites obtained were assigned as rapidly reacting lipase molecules.

Lyophilised preparations of *Thermomyces lanuginosus* lipase

Lyophilised *Thermomyces lanuginosus* lipase as received from the manufacturer expressed low catalytic activity for the esterification of 1-phenylethanol with caproic acid (Table 1). The specific activity was increased if the enzyme was lyophilised from 20 mM phosphate buffer, and even higher activity was obtained if buffers of higher concentration were used (Table 1). Lyophilisation from 200 mM phosphate buffer caused an activation by a factor of seven relative to the preparation from the manufacturer.

The active site titrations showed that lyophilisation from phosphate buffer increased the number of rapidly reacting sites considerably, and the numbers increased with increasing phosphate concentration. The number of slowly reacting sites was not determined, as discussed above, so the numbers in Table 1 only give the lower limit for the fraction of active lipase molecules in the different lyophilised preparations. Likewise, the specific activities per titrated active site are upper limits, which neglect the contributions from the slowly reacting lipase molecules. The fact that the highest values of specific activities per titrated active site were found for those preparations with the lowest numbers of rapidly reacting sites indicates that the

Table 1. *Specific activity and the amount of active sites (% of protein content) for freeze-dried and immobilised lipase from* Thermomyces lanuginosus.[a]

Preparation	Protein loading [mg per g of preparation]	Specific activity[b] [U per g of protein]	Active sites accessible [%]	Specific activity[b] [U per g of active enzyme]	Specific activity[c] [U per g of protein]	Activity ratio[d]
EP-100	55	7.2	93	7.7	7344	1020
EP-100	6.6	3.0	70	4.2	n.d.[e]	n.d.[e]
200 mM phosphate	100	0.12	≥ 50[f]	≤ 0.24	9.5	79
100 mM phosphate	209	0.089	≥ 46[f]	≤ 0.19	4.9	55
20 mM phosphate	630	0.05	≥ 13[f]	≤ 0.38	2.2	44
crude	700	0.017	≥ 3.5[f]	≤ 0.49	n.d.[e]	n.d.[e]

[a] The titrations were performed for 24 h in hexane/diethyl ether (97:3) at a water activity (a_w) = 0.33. The *p*-nitrophenol released was extracted with 200 mM phosphate buffer (pH 7.0) in the case of freeze-dried preparations and with 50 mM Tris-HCl (pH 8.0) in the case of immobilised preparations. The specific activity was measured in the esterification of 50 mM 1-phenylethanol with 100 mM caproic acid or in the esterification of 50 mM dodecanol with 100 mM decanoic acid in hexane at $a_w = 0.33$. [b] Esterification of 1-phenylethanol. [c] Esterification of dodecanol. [d] Ratio in specific activity between dodecanol/1-phenylethanol esterification. [e] n.d. = not determined. [f] Probably not the final value.

slowly reacting ones indeed contributed to the catalytic activity, although to a smaller extent than the rapidly reacting ones.

The positive effect of phosphate buffer on lyophilisation can be explained in different ways. At the highest buffer concentration, the preparation contained only 100 mg of protein per gram. This means that the buffer salts can be regarded as a support material on which the lipase is immobilised. Under suitable conditions, immobilisation can reduce mass transfer limitations relative to those in pure protein powders, and this might have been the mechanism active here. Similar positive effects of salts have previously been reported for co-lyophilisation of lipase and KCl.[9, 11] In that case, the rapidly reacting lipase molecules would be those on the surface of the preparation (with the specific surface area increasing with increasing phosphate concentration). Alternative explanations are that a high amount of phosphate salts in the preparation might favour the open conformation of the lipase and thereby cause a high activity, that the phosphate buffer might act as a lyoprotectant for the enzyme during the freeze-drying procedure or that the buffer might protect the enzyme from direct inactivation by the solvent. In order to investigate the situation further, the same lipase preparations were used to catalyse a much faster reaction, the esterification of dodecanol with decanoic acid. The ratio between the specific activities in the two reactions was considerably higher for the preparation with the highest amount of phosphate than for the one with the lowest salt content (Table 1). This shows that, in the fast reaction, mass transfer at least limited the rate of the preparations with the lower amounts of phosphate.

Thermomyces lanuginosus lipase immobilised onto porous polypropene

When enzymes are immobilised on porous supports and used in organic media, the specific activity observed depends on the enzyme loading (mg of enzyme per g of preparation).[20, 28, 29] At low loadings, low specific activity has been described as being due to partial enzyme inactivation, whilst at high loadings, mass transfer limitations can decrease the specific activity. In the current study, the specific activity at a loading of 55 mg g^{-1} was considerably higher than that at 6.6 mg g^{-1} (Table 1). Active site titration showed that at the high loading almost all the active sites were accessible and reacted rapidly with the reagent. At the lower enzyme loading, only 70% of active sites were titrated, which indicates inactivation of a considerable fraction of the enzyme molecules. Furthermore, the activity per titrated active site was lower at low enzyme loading than at the high loading levels. The low activity obtained at low loading levels is due to direct inactivation of the enzyme by the support, which is diminished at higher protein loadings due to favourable protein – protein interactions.[30]

On comparison of the lyophilised and the immobilised preparations, it is obvious that immobilisation on polypropylene results in preparations with both a higher percentage of titrated active sites and higher activity per active site. The fraction of titrated active sites varied by a factor of about 2 between the best immobilised preparation and the best lyophilised preparation (from 200 mM phosphate), while the activity per titrated active site varied by a factor of more than 30. The differences between the activities per active site may be due to the enzyme being in a favourable conformation when immobilised, or to the lyophilised preparations suffering from severe mass transfer limitations. It is of considerable interest to evaluate the relative importance of these two rate-reducing mechanisms. Recent studies on freeze-dried proteins have provided important information on the mass transfer conditions in those preparations.[31] The accessibility of myoglobin in freeze-dried preparations and when immobilised on silica or EP-100 was investigated by chemical modification with acyl chlorides.[31] Freeze-dried preparations had much slower modification rates compared to when the protein was immobilised onto silica or EP-100. It was suggested that the freeze-dried preparations suffer from very slow solid-phase diffusion. In the current study, the existence of mass transfer limitations was evaluated by use of reactions with different intrinsic rates. When the faster esterification reaction was carried out with the immobilised preparation, the specific activity was more than 1000 times higher than in the slow esterification reaction (Table 1). Since the ratio between the specific activities in the two reactions was much lower for the lyophilised preparations, it is clear that the fast reaction in the lyophilised preparations was severely slowed by mass transfer limitations.

Mass transfer effects have previously been shown to decrease the effective stereospecificity of lipase-catalysed reactions.[32] It is

thus likely that mass transfer limitations influenced the relative rates of the conversion of the two enantiomers of 1-phenylethanol. However, much more extensive mass transfer limitations were demonstrated by the use of the intrinsically fast esterification of 1-dodecanol.

Water-activity dependence of immobilised lipases

It is well known that the catalytic activity of enzymes in an organic medium depends on the water activity of the medium. Often, an increase in catalytic activity with increasing water activity is explained in terms of increased internal flexibility of the enzyme.[2, 33] Water is supposed to act as a "lubricant". Lipases, however, show very different water-activity profiles.[34, 35] Lipase from *Burkholderia cepacia* behaves like most other enzymes, expressing increased activity with increasing water activity.[35] Lipases from *Candida rugosa* and *Rhizopus arrhizus*, however, have bell-shaped water-activity profiles. The bell-shaped profiles were to some extent caused by an increase in the K_m (Michaelis constant) value of the alcohol at higher water activities in the lipase-catalysed esterification reaction.[35, 36]

Graphs showing specific activity as a function of water activity (Figure 2 A and B) for the two lipases studied agree well with previous results on lipases.[35, 37] Active site titrations of all the preparations resulted in graphs very similar to the activity graphs (Figure 3 A and B). To ascertain whether titrations (after 24 h) were complete, the residual activity for the esterification of 1-phenylethanol was measured after the titration. At all water activities, the residual activity was $< 3\,\%$. Longer titration times of up to 72 h were also applied, and the number of active sites at the different water activities did not increase as the titration times were prolonged; this indicates that the active site titration was complete after 24 hours at all water-activity levels. The results indicate that the specific lipase activity per active enzyme molecule at the different water activities was independent of the water activity and that the variation in activity was instead due to differences in the fraction of active enzyme molecules at

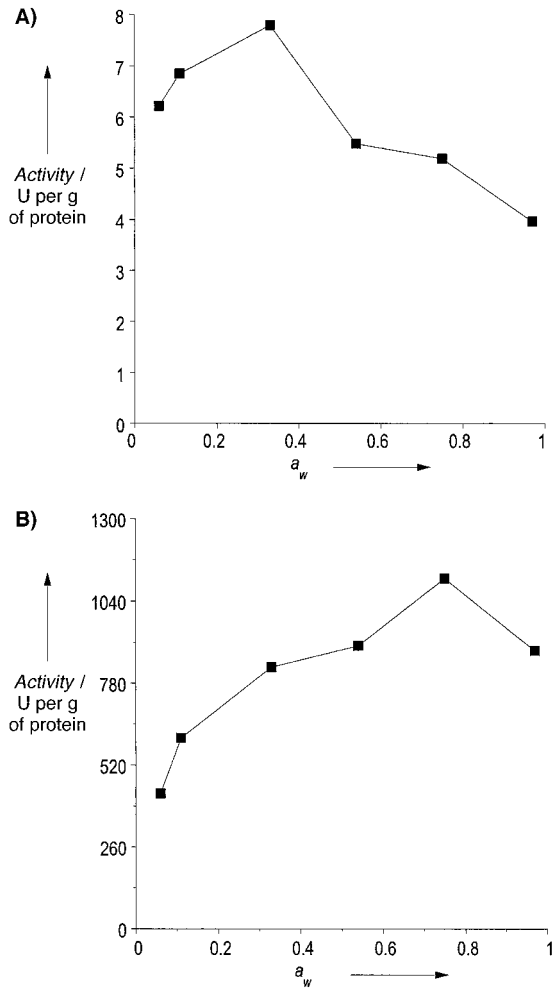

Figure 2. *Water-activity profiles for lipases from A)* Thermomyces lanuginosus *and B)* Burkholderia cepacia. *The reaction was the esterification of 50 mM 1-phenylethanol with 100 mM caproic acid in hexane. The loading on Accurel EP-100 was 55 mg g^{-1} for* Thermomyces lanuginosus *and 36 mg g^{-1} for* Burkholderia cepacia. *The errors in the activity measurements were $< 5\,\%$.*

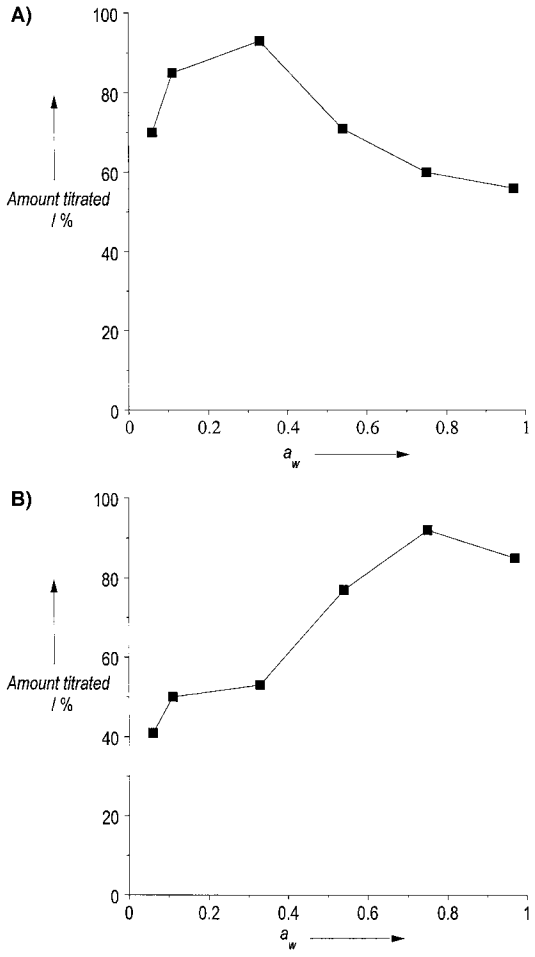

Figure 3. *Amount of titrated enzymes (% of protein content) of A)* Thermomyces lanuginosus *and B)* Burkholderia cepacia *lipases immobilised onto Accurel EP-100 at different water activities. The loading on Accurel EP-100 was 55 mg g^{-1} for* Thermomyces lanuginosus *and 36 mg g^{-1} for* Burkholderia cepacia. *The active site titrations were performed for 24 h in hexane/diethyl ether (97:3). The concentration of the inhibitor MNPHP was 0.36 mM. The* p*-nitrophenol released was extracted with 50 mM Tris-HCl (pH 8.0). The errors in the active site titrations were $< 5\,\%$.*

different water activities. It can be assumed that the decrease in active enzyme molecules at low water activity may be due to reversible conformational changes.[33] The enzyme can be "trapped" in an inactive conformation. It has often been observed that inactivation of this kind can be reversed by increasing the degree of hydration of the enzyme.[33, 38]

The decreased activity at high water activities may be due to diffusional limitations. It can be speculated that the substrate has to diffuse through a small region of aqueous phase surrounding the enzyme particles, which might limit the overall reaction rate at high water activity. If this were the case, a higher intrinsic catalytic rate (obtained by, for example, a change to a faster reaction) would result in a smaller increase in the observed reaction rate at high water activity than at low water activity. The water-activity profiles for *Thermomyces lanuginosus* lipase, however, were similar for the esterifications of 1-phenylethanol and of dodecanol (Figure 2 A and Figure 4), which display a 1000-fold difference in their esterification rates. This indicates that diffusion limitations are probably not the cause of decreased enzyme activity at high water activities.

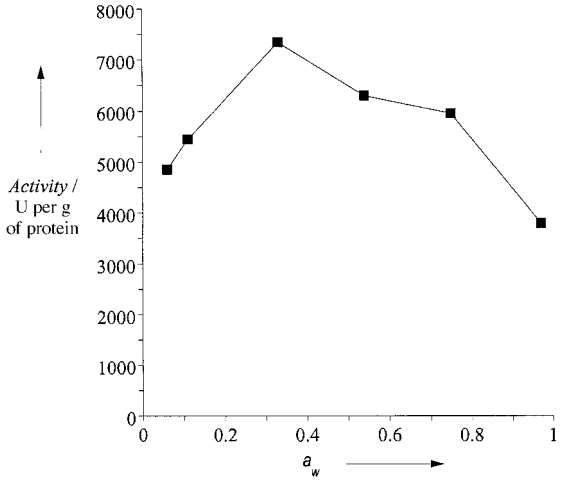

Figure 4. *Water-activity profile for lipase from* Thermomyces lanuginosus. *The reaction was the esterification of 50 mM dodecanol with 100 mM decanoic acid in hexane. The loading on Accurel EP-100 was 55 mg g^{-1}. The errors in the activity measurements were <5 %.*

Instead, the decreased number of active sites at higher water activities for lipase from *Thermomyces lanuginosus* may be partly due to enzyme inactivation during the water-activity equilibration. There are several reports showing that enzyme inactivation is faster at high water activities/water contents than at lower water activities.[39–41]

The fact that changes in catalytic activity and fraction of titratable active sites correlate so well implies that the catalytic activity per competent active site is constant over the whole range of water activities. Studies comparing active site titration data with catalytic activities in organic solvents have previously mainly been limited to subtilisin.[42–44] Those studies show that the catalytic activity per competent subtilisin active site (expressed as k_{cat}/K_m) varied considerably with the water content of the solvent. When the water content in tetrahydrofuran was increased from 0 to 5 μL mL^{-1} the k_{cat}/K_m value increased six- and tenfold for lyophilised subtilisin Carlsberg[44] and subtilisin BPN′.[42] This indicates that there is a fundamental difference between subtilisin and the lipases used in this study and confirms previous observations that lipases are very well adapted for catalysis at low water activities.[6, 45]

Experimental Section

Chemicals and enzymes: Hexane, diethyl ether (>99.5 %), ethyl acetate, dichloromethane, pentane (99 %), methanol, caproic acid (99 %) and silica gel (Kieselgel 60, 230–400 mesh) were purchased from Merck (Darmstadt, Germany). Polypropylene powder, Accurel EP-100 (200–400 μm), was a gift from Akzo (Obernburg, Germany). 1-Phenylethanol (99 %), 1 H-tetrazole, hexylphosphonic dichloride, dodecanol (99 %), decanoic acid (99–100 %) and *p*-nitrophenol (spectrophotometric grade) were purchased from Sigma (St. Louis, USA). Other chemicals used were of analytical grade. The solvents were dried over molecular sieves (pore diameter 3 Å; Sigma, St. Louis, USA).

Lipase from *Thermomyces lanuginosus* (SP 463, HLL) was kindly donated by Novo Nordisk A/S (Bagsvaerd, Denmark), and lipase from *Burkholderia cepacia* (Lipase PS, PCL) was a gift from Amano Pharmaceutical Co. (Nagoya, Japan).

Immobilisation by adsorption: Lipases from *T. lanuginosus* and *B. cepacia* were immobilised on macroporous polypropylene, Accurel EP-100. Lipase powder was dissolved in phosphate buffer (20 mL, 20 mM, pH 6.0) and centrifuged. The supernatant was added to Accurel EP-100 (1 g), which was prewetted with ethanol (3 mL per g of support). The support and the enzyme solution were incubated overnight at room temperature and then filtered. Phosphate buffer (200 mM, pH 7.0) was added, and the preparation was dried overnight under reduced pressure. The amount of protein used per gram of Accurel EP-100 in the adsorption was 36 mg for *B. cepacia* lipase and 55 mg or 6.6 mg for *T. lanuginosus* lipase.

Freeze-dried preparations: Lipase from *T. lanuginosus* (120 mg) was suspended in phosphate buffer (16 mL, 20–200 mM, pH 7.0); any solid particles present were removed by centrifugation. The supernatant was immediately frozen at −80 °C and then freeze-dried for 24 h.

Protein determinations: The protein contents of the crude lipase powders and the freeze-dried preparations were determined by the Bradford method,[46] with BSA (bovine serum albumin) as a standard. The amount of lipase adsorbed onto Accurel EP-100 was determined by measuring the amount of protein in the solution before and after adsorption had occurred. In all cases, 100 % of the proteins were adsorbed onto Accurel EP-100.

Water-activity adjustments: The enzyme preparation (immobilised or freeze-dried) and the solvent (hexane) or substrate solution (50 mM alcohol, 100 mM acid in hexane) were incubated overnight over saturated salt solutions to a defined initial a_w prior to the titration or the esterification reaction.[35] The salts used for equilibration were LiBr ($a_w = 0.06$), LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $MgNO_3$ ($a_w = 0.54$), NaCl ($a_w = 0.75$) and K_2SO_4 ($a_w = 0.97$).

Synthesis of methyl *p*-nitrophenyl *n*-hexylphosphonate (MNPHP): The synthesis of the active site inhibitor MNPHP was performed according to a previously described method.[22] The MNPHP was purified by flash column chromatography over silica with EtOAc/CH_2Cl_2 (1:9) as the eluent. The yield of purified (>98 %) MNPHP was 35 %. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.90$ (t, $J = 6.9$ Hz, 3 H; CH_2CH_3), 1.29–1.35 (m, 4 H), 1,41 (m, 2 H), 1.65–1.73 (m, 2 H) 1.90–2.00 (m, 2 H;

P–CH_2), 3.82 (d, J(H,P) = 11.2 Hz, 3H; O–CH_3), 7.40 (d, J = 9.2 Hz, 2H; Ph(2,6)), 8.27 (d, J = 9.0 Hz, 2H; Ph(3,5)); ^{31}P NMR (300 MHz, $CDCl_3$): δ = 32.2.

Active site titration: Before the active site titrations were started, the enzyme preparation and the solvent were equilibrated to a defined water activity (as described above). In a 2-mL glass vial, hexane (640 μL) and MNPHP (20 μL, 12 mM in diethyl ether) were added to the enzyme preparation. The reaction mixture was incubated at 25 °C. The *p*-nitrophenol was extracted from the organic phase with Tris-HCl buffer (1 mL, 50 mM, pH 8.0), with an extraction yield of 77%. The aqueous solution was centrifuged and the amount of *p*-nitrophenol was measured spectrophotometrically at 400 nm on a Shimadzu UV-120-02 apparatus (molar extinction coefficient = 15 700 $M^{-1}cm^{-1}$).

In the case of freeze-dried preparations, the *p*-nitrophenol was extracted with phosphate buffer (1 mL, 200 mM, pH 7.0). The freeze-dried preparation dissolved in the aqueous phase during the extraction. Phosphate buffer at pH 7.0 was therefore used to avoid pH changes in the aqueous solution during the extraction, which might result in alteration of the molar extinction coefficient. The extraction yield in this case was 75% and the molar extinction coefficient was 7000 $M^{-1}cm^{-1}$.

For every active site determination, 4–5 titrations were performed with different amounts of enzyme preparation (0–3 mg of protein). The amount of *p*-nitrophenol released was plotted against the amount of preparation, and the slope obtained gave the amount of titrated active sites per mg of preparation. The amount of titrated lipase was calculated by using molecular weights of 32 kDa for *T. lanuginosus*[47] and 33 kDa for *B. cepacia*.[48] The ratio of titrated lipase and total protein (mg mg^{-1}) was plotted in the graphs.

Esterification reactions: Before the reactions were started, the enzyme preparation and the substrate solution were equilibrated to a defined water activity (as described above). The reactions were performed in hexane at 25 °C in 4-mL screw-capped vials with Teflon-lined septa and started by addition of enzyme preparation to the substrate solution (2 mL containing 50 mM alcohol and 100 mM acid). Reaction vials were shaken on a horizontal shaker (175 rpm). Samples (50 μL) were withdrawn at different degrees of conversion, diluted in hexane (450 μL) and analysed by gas chromatography.

Measurement of residual activity after inhibition: Investigation of the residual activity of the inhibited enzyme preparation was performed according to the following procedure. The hexane/diethyl ether mixture (97:3) was removed from the inhibited enzyme preparation. The preparation was washed twice with pentane (1 mL) and dried in a desiccator. The residual activity was assayed by the esterification reaction described above.

GC analysis: The alcohol substrates (dodecanol or 1-phenylethanol) and the ester products were analysed on a Shimadzu gas chromatograph (GC-14 A) equipped with a flame ionisation detector and a column packed with GP 10% SP-216-PS on Supelcoport. Helium was used as the carrier gas. The temperature of the injector was 200 °C and of the detector 240 °C. The degree of conversion was calculated from the relative amounts of ester and alcohol, and the initial reaction rates were calculated.

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