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Molecular Imprinting of Enzymes with Water-Insoluble Ligands for Nonaqueous Biocatalysis

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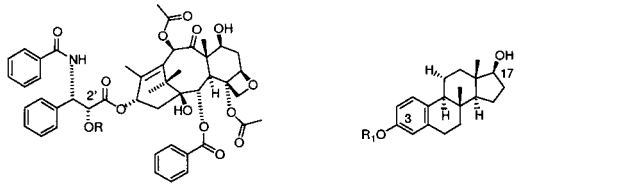
Attaining higher levels of catalytic activity of enzymes in organic solvents is one of the major challenges in nonaqueous enzymology.[1] One of the most successful strategies for enhancing enzyme activity in organic solvents involves tuning the enzyme active site by molecular imprinting with substrates or their analogues.[2] Molecular imprinting is achieved by dissolving an enzyme in the presence of a ligand ("imprinter") followed by freeze-drying the solution. The resultant lyophilized enzyme−imprinter complex presumably remains "trapped" in its active conformation and therefore retains higher activity in the nonaqueous environment as compared to the nonimprinted enzyme.[2] Implementation of this approach requires the imprinter to be freely soluble in the aqueous solution from which the enzyme−ligand complex is lyophilized. Unfortunately, numerous imprinters of potential importance (e.g., many natural products) are poorly soluble in water, which significantly limits the utility of this method.

In the present study, we have developed strategies that overcome this limitation of the molecular imprinting technique and that thus expand its applicability beyond water-soluble ligands. We have also demonstrated that the activation effects of molecular imprinting and co-lyophilization with inorganic salt are additive, and therefore the combination of these strategies can dramatically enhance enzyme activity in organic solvents.

In a previous study we demonstrated that thermolysin catalyzed the regiospecific acylation of paclitaxel (**1**) at the 2′-position (Scheme 1) in anhydrous *tert*-amyl alcohol.[3] In other studies it was shown that the protease subtilisin[4] and microbial lipases[5] can catalyze the selective acylation of a wide variety of steroids, including 17β-estradiol (**3**). Because the aqueous solubilities of **1** and **3** are extremely low (0.7 μg/mL[6] and 2.5 μg/mL,[7] respectively), we chose the thermolysin/paclitaxel, subtilisin Carlsberg/17β-estradiol and lipase TL from *Pseudomonas stutzeri*/17β-estradiol pairs as convenient models for exploring strategies to achieve imprinting with water-insoluble ligands. As expected, an attempt to imprint thermolysin directly with **1** by simply adding **1** to an aqueous enzyme solution prior to lyophilization did not result in an increase of thermolysin activity in the acylation of **1** with divinyl adipate in anhydrous *tert*-amyl alcohol. Similarly, no activation of subtilisin or lipase TL was observed in acylation of **3** with vinyl butyrate in anhydrous acetone upon lyophilization of the enzymes from an aqueous buffer containing **3**. Presumably, due to the low solubility of **1** and **3** in water the concentration of the imprinters in the lyophilization medium was not high enough to afford an effective complex with the enzyme and thus produce the imprinting effect.

Scheme 1



1: R=H (paclitaxel)
2: R=CO(CH$_2$)$_4$COOH (paclitaxel-2′-adipic acid)

3: R$_1$=H (17β-estradiol)
4: R$_1$=SO$_3$ (17β-estradiol 3-sulfate)
5: R$_1$=CH$_2$COOH (17β-estradiol 3-carboxymethyl ether)

The solubility problem can be addressed either by modifying the imprinter to increase its water solubility or by modifying the medium to increase its ability to dissolve the hydrophobic imprinter. We have succeeded in applying both of these strategies to produce imprinted thermolysin, subtilisin, and lipase TL possessing improved catalytic activity in the acylation of **1** and **3**.

In the first approach, we used paclitaxel-2′-adipic acid (Scheme 1, compound **2**) as a water-soluble imprinter. This paclitaxel derivative, which is ca. 1700-fold more soluble in water than native **1**, was synthesized enzymatically using our previously reported method.[3] The catalytic activity of thermolysin imprinted with **2** increased 8.9-fold in anhydrous *tert*-amyl alcohol as compared to the nonimprinted enzyme (Table 1, entry 2), with no change in the regioselectivity of pacitaxel acylation. Imprinting subtilisin and lipase TL with hydrophilic estradiol derivatives **4** and **5**, which have ca. 8000- and 800-fold higher aqueous solubility than **3**, respectively, also proved to be successful for attaining high catalytic activity for these enzymes in anhydrous acetone. For example, subtilisin imprinted with **4** showed a 10-fold increase in catalytic activity (Table 1, entry 13), while imprinting of lipase TL with **4** and **5** resulted in 15- and 26-fold activation, respectively (Table 1, entries 17 and 18).

In an alternative approach, enzymes were dissolved in aqueous solution containing unmodified imprinter (**1** or **3**) and 30% (v/v) *tert*-butyl alcohol or 1,4-dioxane. Due to the presence of the organic solvent the solubility of imprinters in the lyophilization medium sharply increased, reaching the level that is generally used for enzyme imprinting (1−5 mg/mL).[2] The solution was then lyophilized, which was possible due to the ability of *tert*-butyl alcohol and 1,4-dioxane to freeze-dry under the same conditions that are normally used for aqueous solutions.[8] Thermolysin treated in this way was activated 3.5-fold (lyophilization from *tert*-butyl alcohol) or 4.1-fold (lyophilization from 1,4-dioxane) as compared to nonimprinted thermolysin (Table 1, entries 6 and 11, respectively). It is important to point out that the presence of *tert*-butyl alcohol

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Table 1. Activation of Enzymes by Lyophilization from Aqueous Buffer and Organic Solvent−Water Mixtures in the Presence of Different Excipients

entry	enzyme	lyophilization medium	excipient	reaction[a]	initial rate v_0, μmol (mg enzyme)$^{-1}$ h^{-1}	activation factor[b]
1	thermolysin	100% buffer	none	A	34	1.0
2	thermolysin	100% buffer	**2**	A	300	8.8
3	thermolysin	100% buffer	98% KCl[c]	A	680	20
4	thermolysin	100% buffer	**2** + 98% KCl[c]	A	3800	110
5	thermolysin	30% (v/v) *tert*-butanol	none	A	35	1.0
6	thermolysin	30% (v/v) *tert*-butanol	**1**	A	120	3.5
7	thermolysin	30% (v/v) *tert*-butanol	98% KCl[c]	A	460	14
8	thermolysin	30% (v/v) *tert*-butanol	**1** + 98% KCl[c]	A	1400	41
9	thermolysin	100% buffer	none	B	37	1.0
10	thermolysin	30% (v/v) 1,4-dioxane	none	B	15	0.41
11	thermolysin	30% (v/v) 1,4-dioxane	**1**	B	150	4.1
12	subtilisin	100% buffer	none	C	54	1.0
13	subtilisin	100% buffer	**4**[d]	C	540	10
14	subtilisin	30% (v/v) *tert*-butanol	none	C	75	1.4
15	subtilisin	30% (v/v) *tert*-butanol	**3**[d]	C	540	10
16	lipase TL	100% buffer	none	C	4.6	1.0
17	lipase TL	100% buffer	**4**[d]	C	68	15
18	lipase TL	100% buffer	**5**[d]	C	120	26
19	lipase TL	30% (v/v) *tert*-butanol	none	C	5.2	1.1
20	lipase TL	30% (v/v) *tert*-butanol	**3**[d]	C	28	6.1

[a] Reaction A is transesterification of **1** (3 mM, ICN) and divinyl adipate (80 mM, TCI America) in *tert*-amyl alcohol; B is transesterification of **1** (3 mM) and vinyl butyrate (80 mM, TCI America) in *tert*-amyl alcohol; C is transesterification of **3** (19 mM, Sigma) and vinyl butyrate (95 mM) in acetone. [b] Activation factor is $(v_0)_{\text{excipient}}/(v_0)_{\text{none}}$. The denominators of this expression are the initial rates given in entries 1 (reaction A), 9 (reaction B), 12 (reaction C with subtilisin), and 16 (reaction C with lipase TL). [c] KCl content is calculated based on the total weight of solids in the enzyme preparation. [d] Subtilisin catalyzes the selective acylation of 17β-estradiol at the C-17 hydroxyl group (Scheme 1).[4] The product of the reaction catalyzed by lipase TL is chromatographically indistinguishable from the 17-acylated estradiol produced by subtilisin, indicating that both compounds are identical.

alone (i.e., without added **1**) in the lyophilization medium had essentially no effect on the activity of lyophilized enzyme (Table 1, entry 5). Moreover, imprinting from 1,4-dioxane resulted in a more active enzyme despite the fact the this solvent had a detrimental effect on enzyme activity when added to the lyophilization solution in the absence of the imprinter (Table 1, entry 10). Thus, the observed activation is not caused by the influence of the solvent on the enzyme during lyophilization, but rather reflects a genuine imprinting effect by **1**. This represents the first report on enzyme imprinting with a ligand as large as **1**.

The generality of the imprinting strategy based on the use of organic solvent to increase the solubility of hydrophobic imprinters in the lyophilization medium was confirmed using subtilisin and lipase TL. In particular, subtilisin and lipase TL imprinted with **3** from 30% *tert*-butyl alcohol showed 10-fold (Table 1, entry 15) and 6.1-fold (Table 1, entry 20) activation, respectively, compared to nonimprinted enzymes. Similar to thermolysin, the presence of *tert*-butyl alcohol alone in the lyophilization buffer did not cause any major change in activity for subtilisin or lipase TL (Table 1, entries 14 and 19, respectively).

In our previous study we found that the activity of thermolysin in the acylation of **1** with divinyl adipate was significantly increased when the enzyme was lyophilized in the presence of a high concentration of KCl.[3] Therefore, we investigated whether salt activation[9] and molecular imprinting[2] can be used together to further activate thermolysin in anhydrous organic solvents. To that end, we lyophilized thermolysin from aqueous buffer in the presence of KCl to produce a catalyst containing 98% of the inorganic salt. The enzyme activity in anhydrous *tert*-amyl alcohol was increased 20-fold compared to thermolysin lyophilized without KCl (Table 1, entry 3), in agreement with our previous results.[3] The addition of the water-soluble paclitaxel derivative to the lyophilization medium resulted in the increase of the activation effect to 110-fold (Table 1, entry 4), which is significantly higher than activation factors obtained when either molecular imprinting or salt addition were applied separately. Similar additive activation was observed when the alternative procedure for enzyme imprinting with the water-insoluble ligand employing *tert*-butyl alcohol as a cosolvent was used in conjunction with KCl. In this case, the combined effect of both excipients was 41-fold, as compared to 3.5-fold activation by cosolvent-assisted imprinting and 14-fold activation by KCl (Table 1, entries 8, 6, and 7, respectively). That imprinting and salt activation effects are additive suggests that they have different mechanisms of action.

In conclusion, we have developed a practical strategy that broadens the scope of molecular imprinting of enzymes to include water-insoluble ligands, either by converting the ligands into a water-soluble form or by adding organic cosolvents to increase their solubility in the lyophilization medium. Furthermore, we have demonstrated for the first time that molecular imprinting and salt activation, applied in combination, produce a strong additive activation effect, suggesting different mechanisms of action involved in these enzyme activation techniques.

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Supporting Information Available: Experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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