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The effect of counterion, water concentration, and stirring on the stability of subtilisin BPN′ in organic solvents [1]

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Abstract

The stability of subtilisin BPN′ in organic solvents or cosolvent/water mixtures was studied as a function of the type and concentration of counterion at the time of freeze-drying, water concentration, and stirring speed/method. It was found that the enzyme is stabilized by high concentrations of counterion, at least at very high cosolvent concentrations. The type of counterion also has a remarkable impact on the enzyme stability; at high concentrations of DMF (dimethylformamide), multivalent counterions with low solubility in organic solvents are far superior to monovalent, soluble ones. Sodium citrate is the best salt tested in terms of enzyme stability, increasing the half life of the enzyme better than a millionfold over Tris in 99% DMF. The stability of the enzyme was found to have a complex dependence on the amount of water in the DMF. Enzyme lyophilized from the sodium phosphate displays a stability minimum at about 90% DMF, while enzyme lyophilized from Tris becomes increasingly unstable from 30% to 99% DMF, without inflection. Vigorous stirring with a magnetic stir bar, which broke apart the enzyme particles, was found to be extremely deleterious to enzyme stability, while swirling the enzyme with a wrist-action stirrer, which did not grind the enzyme particles, had no effect. Explanations for this are discussed.

Keywords: Counterion; Water concentration; Subtilisin BPN′

1. Introduction

The efforts of a number of groups have been focused on the use of enzymes suspended in organic solvents or dissolved in water-miscible organic cosolvent + water mixtures to accomplish organic transformations such as peptide and glycopeptide coupling [1–4], regiospecific acylation of sugars [5,6], enantioselective resolutions, [7–9], transesterifications [10,11], glycosidase-catalyzed polysaccharide synthesis [12,13], and others. The organic solvents are often required to enhance substrate solubility and to minimize side reactions or drive the reaction equilibrium in a preferred direction. However, the stability and activity of enzymes typically drop greatly in solutions containing large amounts of organic cosolvent; therefore, efforts have been made to stabilize enzymes toward these conditions via mutagenesis [14,15], and to activate them by addition of salts [16], lyoprotectants such as sugars or polyethylene glycol [17], or other additives. There has been

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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speculation that some of the 'activating' effects of salts may actually be due to *stabilization* of the enzyme toward lyophilization and/or solvent-induced denaturation [16,17]. The effects of counterion, stirring, and water concentration on the stability of enzymes in organic solvents have not been thoroughly investigated, although they have been studied with regard to enzymatic activity. Dordick [18] observed a higher activity for Subtilisin Carlsberg when lyophilized from potassium chloride than when lyophilized from sodium chloride; Affleck et al. [19] have reported that the activity of the same enzyme shows a complex dependence on water concentration. Although many groups have studied the effect of buffer type and pH on the stability of enzymes in aqueous media, no group to date has published a thorough study on the dependence of protein stability on these parameters when the enzyme is suspended in solvent.

We originally became interested in this topic when searching for mutants of subtilisin that were stabilized toward organic solvent-mediated denaturation by removing surface charges [20]. To our surprise, we discovered that the enzyme was far more sensitive to the lyophilization and suspension conditions than to the mutations we had made. In our preliminary study of the effect of various parameters on the stability of subtilisin in DMF, we discovered that virtually *every parameter we tested*, including counterion type and concentration, water concentration, and even the manner of stirring, had a profound effect on the stability of the enzyme [20]. In this paper, we follow up those studies with a more systematic investigation of these phenomena.

2. Results

As noted in the paper cited above [20], we discovered that the concentration of buffer salt lyophilized with the enzyme had a pronounced effect on the stability, at least to point. Fig. 1a and b show plots of the stability of subtilisin BPN′ in 99% and 90% dimethylformamide

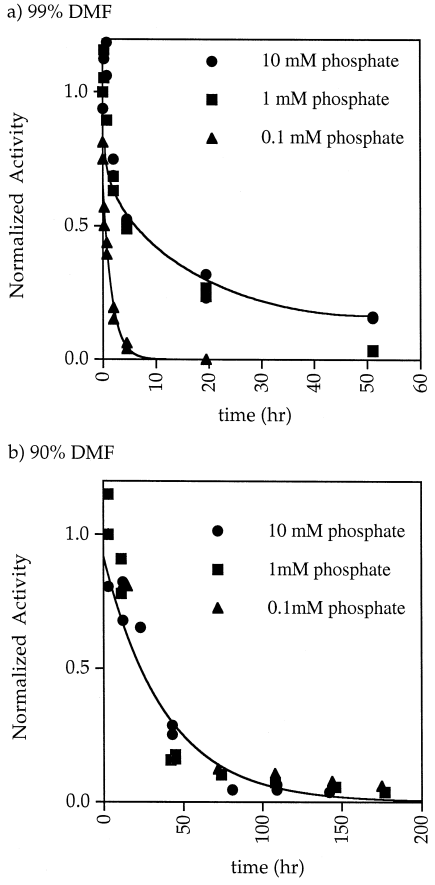

Fig. 1. Stability of subtilisin BPN′ (0.01 mM) lyophilized from 10, 1, or 0.1 mM sodium phosphate, pH 7.0, +0.025 mM $CaCl_2$ in (a) 99% DMF, 25°C; or (b) 90% DMF, 25°C.

(DMF), respectively, when a ~0.01 mM solution of enzyme was lyophilized in the presence of 10, 1, and 0.1 mM sodium phosphate, pH 7.0 + 0.025 mM $CaCl_2$. Calcium is included in all studies, since subtilisin is known to have two calcium binding sites, and the stabilizing effect of calcium on subtilisin and a number of other proteases is well established. DMF was chosen as the solvent because the peptide substrates for subtilisin are soluble in it, but sparingly soluble in more hydrophobic organic solvents where many enzymes have typically better activity and stability. We have noticed that subtilisin also has much better stability in more hydrophobic solvents such as ethyl acetate and hexane, but unfortunately polypeptides are typically minimally soluble in these solvents.

In 99% DMF, subtilisin has essentially identical stabilities when lyophilized from the top two phosphate concentrations, but the stability of enzyme prepared from the lowest concentration of buffer is diminished by an order of magnitude (Fig. 1a). Not shown on the graph is the data for enzyme lyophilized from extensively dialyzed enzyme (minimal phosphate concentration). In this condition, the enzyme deactivates almost instantaneously in solvent; no activity could be measured within 1–2 s of the addition of DMF. In 90% DMF (Fig. 1b), however, all three preparations had equivalent half-lives. All further stability studies were conducted with enzyme prepared with approx. 1000:1 molar ratio of buffer salt:enzyme (samples were lyophilized from 10 mM salt + ~0.01 mM enzyme).

We next tested the stability of the enzyme as a function of the type of buffer salt used. For these studies, we investigated a variety of inorganic (sodium bicarbonate, sodium and potassium phosphate, and sodium borate) and 'mixed' (organic + inorganic ion pairs: sodium citrate, Tris–HCl, or the sodium salts of MOPS, HEPES, and MES) buffers. The results of the studies in 90 and 99% DMF are listed in Table 1. The numbers given ($T_{50\%}$) are the times required for the enzyme to decay to half of its original activity. The decays were often biphasic, with a rapid initial drop in activity followed by a much slower decay of the remaining activity, so $T_{50\%}$ does not always represent a half life. For that reason, the % activity remaining at 50 h (for 99% DMF) or 2.6 h (for 90% DMF) is also given. In 99% DMF, the enzyme preparations lyophilized from all of the 'mixed' buffers except sodium citrate were inactivated almost immediately, while those lyophilized from inorganic buffers were usually much more stable (the sole exception being sodium bicarbonate). In 90% DMF, enzyme lyophilized from 'mixed' salts was more stable than in 99% DMF, while that lyophilized from inorganic buffers was less stable. This was unexpected, and so we investigated the stability of subtilisin in DMF with various concentrations of water and either Tris–HCl or sodium phosphate as representative 'mixed' and 'inorganic' counterions. The results are shown in Table 2. The results for subtilisin lyophilized from phosphate are complex. In pure buffer, where hydrolysis is a major factor in deactivation, the enzyme is quickly inactivated, but when 30–70% DMF is added, autohydrolysis diminishes, and the enzyme becomes much more stable. This is not particularly surprising.

Table 1
Deactivation rates for subtilisin BPN′ lyophilized from a 1000:1 molar ratio of various buffers (0.01 mM enzyme, 10 mM buffer + 25 μM $CaCl_2$) and resuspended in 99% or 90% DMF/balance water (25°C, stirring)

	99% DMF		90% DMF	
	$T_{50\%}$	% activity remaining after 50 h	$T_{50\%}$	% activity remaining after 2.5 h
Mixed salts				
Tris–HCl	< 1 min	< 0.1%	7 min	< 0.1%
HEPES	< 1 min	< 0.1%	7 min	5%
MOPS	< 1 min	< 0.1%	N.D.	N.D.
MES	< 1 min	< 0.1%	N.D.	N.D.
Sodium citrate	240 h	76%	N.D.	N.D.
Inorganic salts				
Sodium phosphate	6 h	16%	0.5 h	4%
Potassium phosphate	8 h	35%	0.7 h	20%
Sodium borate	80 h	59%	2.3 h	57%
Sodium carbonate	2 min	< 0.1%	35 min	7%

$T_{50\%}$ is the length of time required for the enzyme to deactivate to 50% of its original activity. In many cases, decay is not exponential, thus this is not necessarily a half life (N.D. = no data).

Table 2
Deactivation rates for subtilisin BPN′ lyophilized from Tris–HCl, pH 8.0 or sodium phosphate, pH 7.0, and resuspended and stirred in DMF + varying amounts of water at 25°C

	Tris–HCl, pH 8		Sodium phosphate, pH 7	
	$T_{50\%}$	% activity remaining after 50 h	$T_{50\%}$	% activity remaining after 50 h
DMF + percent water				
0%	2 h	2.5%	7.5 h	< 0.1%
30%	> 150 h	90%	> 150 h	100%
50%	> 150 h	100%	> 150 h	92%
70%	> 150 h	100%	30 h	35%
80%	4 h	13%	1 h	2.3%
90%	5 min	< 0.1%	1 h	8%
99%	1 min	< 0.1%	7 h	26%

Enzyme (0.01 mM) was lyophilized from 10 mM buffer + 25 μM $CaCl_2$, giving a 1000:1 molar ratio of buffer salt:enzyme.
$T_{50\%}$ refers to the time required for the enzyme to undergo a 50% decay in activity.
In many cases, decay was not exponential, thus this is not necessarily a half-life.

After 70%, though, the stability drops off, reaching a slight minimum at 90%, and then increases again as the DMF concentration rises to 99%. This local minimum is not observed with Tris; the stability of enzyme lyophilized from Tris diminishes without inflection from 80% all the way to 99% DMF. Curiously, at 80% DMF, enzyme prepared with Tris buffer is actually more stable than that prepared with phosphate. We conducted a similar study in ethanol. The plots, shown in Fig. 2, show that again enzyme prepared with phosphate displays a local minimum, this time at about 70% ethanol; however, the stability of enzyme prepared from Tris *increases* steadily as the concentration of ethanol rises from 50 to 90%.

As mentioned in a previous paper [20], stirring was found to have a profound effect on the stability of the enzyme (see Fig. 3a). Stirring with a magnetic stirring bar (at approx. 20–30 Hz), however, breaks up the enzyme particulates, and so it is not clear from that study whether the increase in mass transfer rates from the rapid agitation caused the loss of activity or whether the physical grinding of the particles was responsible. We found that even vigorous swirling, which does not break up the enzyme precipitate, does not have the same effect (Fig. 3b).

We conducted solubility studies (in 99% DMF) on the enzyme lyophilized from Tris or phosphate, too, in case the effect we observed was due to simply the gradual dissolution of the enzyme, which would be accelerated by stirring. In either case, the solubility of the enzyme was negligible (as determined by a Coomassie assay of the supernatant), on the order of 6 ng/ml. It is worth noting that the Tris enzyme appears to

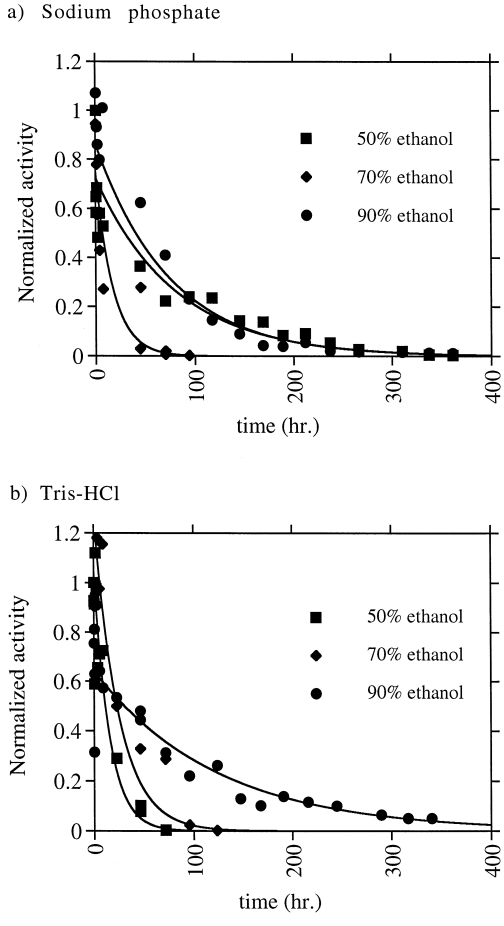

Fig. 2. Stability of subtilisin BPN′ in various concentrations of ethanol at $T = 25$°C, stirred. (a) lyophilized from 0.01 mM enzyme/10 mM sodium phosphate + 25 μM $CaCl_2$, pH 7.0. (b) lyophilized from 10 mM Tris–HCl + 25 μM $CaCl_2$, pH 8.0.

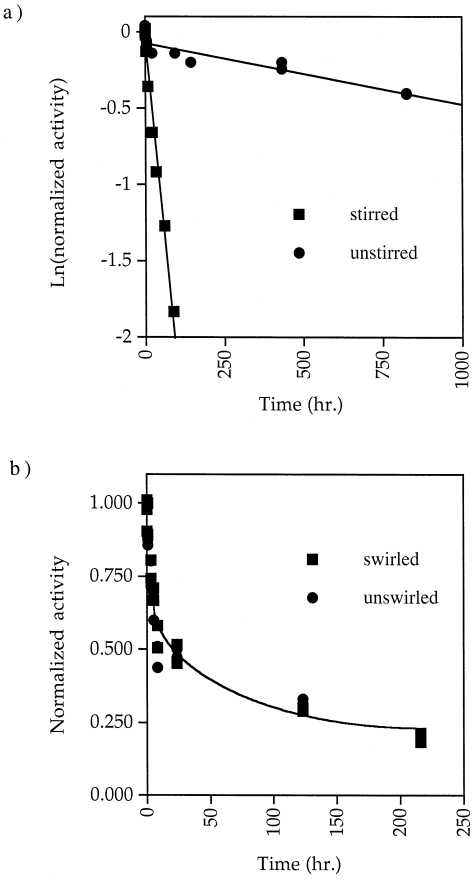

Fig. 3. Effect of stirring on the stability of subtilisin BPN in 99% DMF. Enzyme (0.01 mM) was lyophilized from 10 mM sodium phosphate/25 μM $CaCl_2$, pH 7.0. Graph (a) compares the deactivation rates of a static enzyme suspension with that of a suspension vigorously stirred with a stir bar at approx. 20–30 Hz. Graph (b) compares the deactivation of enzyme that has been pre-ground to a fine powder, then suspended in solvent and either left static or swirled with a wrist action agitator.

be soluble, as noted in our previous paper, since the buffer salt (which forms the majority of the solid) does dissolve, leaving very little solid remaining. Very little protein was actually detected in solution, however.

3. Discussion

The dependence of enzyme stability on the counterion:enzyme ratio is not surprising. It seems likely that the salts would form a counterion layer around the enzyme, shielding it somewhat from the solvent, and that the thinner the layer, the weaker the barrier. What is more remarkable is the effect of the *type* of counterion on the stability of the enzyme. In very high (ca. 90% or more) concentrations of DMF, 'mixed' buffers (containing organic and inorganic ions) are poor salts to use. As the percent DMF (or ethanol) drops, the 'mixed' counterions become more effective and the inorganic ions less effective at stabilizing the enzyme (at least in the case of phosphate and Tris).

These trends could be due to a variety of factors. The 'mixed' buffers are more soluble in solvents than the inorganic ones, and so they may simply dissolve away from the enzyme. In many cases, it was clear that this must be happening, since much of the solid had clearly dissolved but very little enzyme was detectable in solution via coomassie assay of the lyophilized and redissolved supernatant. Differential solubilities of enzyme and buffer salt at the various water/solvent ratios could also explain the complex dependence of enzyme stability on water concentration. Alternatively, the 'mixed' counterions may allow DMF to more easily penetrate the hydrated salt 'shell' which we presume surrounds the enzyme. The effects we observed may stem from the ability of the inorganic salts we used to coordinate more water than the 'mixed' counterions we used. Inorganic salts have been used, in fact, as water 'buffers' for enzymatic reactions in organic solvents [21]. The inorganic salts may therefore provide a better hydrated 'shell,' though sodium citrate, interestingly, was the best counterion we found in either 90 or 99% DMF. We imagine that this effect is due to the 'cross-linking' (via salt-bridges with basic residues) of the enzyme by the trivalent citrate. This could also explain why the enzyme displays high stability when lyophilized from phosphate.

It had occurred to us that the different stabilities observed with different salts could be due to pH effects. Klibanov noted that enzyme activity

is optimal in organic solvents when the enzyme is lyophilized from a buffer at the enzyme's optimal pH in water [22]. This observation was termed 'pH memory,' and works well for phosphate systems. Russell's group extended the study and found that the accuracy of pH memory is dependent on the type of buffer salt used. For some salts, the pH optimum in solvents is displaced from that observed in water [23]. This is not unexpected, since buffers will have altered pK_a's in the presence of solvent, and the direction and magnitude of the pK_a shift depends on the particular salt. (For example, it is known that the pK_a of a carboxylic acid can shift 5 units upon transfer from water to ethanol, while Tris varies little.) It may be possible that the pK_a's of the inorganic buffers we chose vary less when moving from water to an organic solvent than the pK_a's of the 'mixed' buffers we used. However, a recent study by Shubhada and Sundaram [24], shows that at high cosolvent concentrations, enzyme stability is less dependent on pH changes of the buffer than on cosolvent-mediated denaturation. They also tabulated the pH shifts of various buffers as the percent cosolvent is increased, and shows that pH swings of up to 3–5 units can occur for borate and phosphate, while the pH shifts are minimal for tris. Since tris was one of the worst buffers in terms of enzyme stability, neither the pH-shift explanation for the rapid deactivation of enzyme lyophilized from that buffer nor the pH memory explanation can be used to support the observations.

Stirring was found to have a profound effect on the stability of the enzyme (Fig. 3a). While this is a well known phenomenon in water, where the enzyme is soluble, it has generally been attributed either to enzyme shearing (in the case of large, multi-enzyme complexes) or to the fact that when an enzyme solution is stirred vigorously in water, the solution froths and the enzymes unfold and 'spread out' on the surface of the bubbles. A *suspension* of enzyme does not froth, however, (though the grinding may shear the enzyme) and so we did not expect that stirring would have such a large effect on enzyme stability.

Stirring has been observed to affect the *activity* of enzymes (e.g., lipase) in solvent, and this has been attributed to the agglomeration of enzyme and resultant diffusional limitations [25]. While we were not able to control the particle size in these stability assays, the loss of activity we observed after incubation in solvent cannot be attributed to particle coalescence, since all of our activity assays were performed after addition of water which dissolved the enzyme completely.

There are a number of reasons why this effect might be observed. It could be that the stirring releases air bubbles trapped with the particles. However, the enzyme particles are rather dense, not buoyant like one would expect with a great deal of entrapped air. Furthermore, no air bubbles were visible, even under the microscope, so any bubbles would have to be less than about 0.1 μm. Alternatively, there could be mass transfer effects, such as the slow dissolution of enzyme from the surface, which would be accelerated by stirring, or diffusion of DMF through the particle, which would be accelerated by the grinding of the particles to smaller size. It is also possible that the actual grinding of the particles shears the enzyme (though we observed no significant deactivation of the enzyme upon grinding in the absence of solvent), or perhaps the enzyme simply deactivates most quickly at the surface of the particle, and so as the enzyme dust is ground finer, the surface area increases and so more and more enzyme deactivates.

In order to determine whether the effect was due to external mass transfer limitations (slow diffusion of, for example, enzyme or water from the surface of the particle to the bulk fluid), we pre-ground the enzyme in order to get a reasonably fine suspension for reproducible sampling and compared the stability of static vs. swirled enzyme (swirling did not break up the enzyme particles). The results (Fig. 3b) demonstrate that the enzyme is equally stable under both condi-

tions, thus external mass transfer is not an issue, though internal mass transfer may still be at fault. Other groups [16,26] have found minimal barriers to diffusion through lyophilized enzymes particles, but they typically used a lower proportion of salt:enzyme.

We also checked the solubility of the enzyme in DMF after lyophilization from Tris or sodium phosphate. In neither case was the enzyme particularly soluble, dissolving to 6 ng/ml with Tris and 5 ng/ml with phosphate. Thus, enzyme deactivation is not due to the enzyme dissolving and then rapidly denaturing, a conclusion that the dependence on stirring might have suggested.

For whatever reason, the enzyme is far less stable when stirred in such a way that the enzyme precipitate is broken apart, but this instability can be alleviated by agitating in such a way that the enzyme particles remain intact.

It is clear from these results that careful preparation of an enzyme is of paramount importance for insuring its activity in high concentrations of organic cosolvents. Factors that are usually given little thought such as the type of buffer salt and the method of stirring can be the difference between success and failure of the enzymatic reaction.

4. Materials and methods

Subtilisin BPN′ (Protease Type XXVII, or Nagarse) was obtained from Sigma (St. Louis, MO), as was its chromogenic substrate, succinyl Ala-Ala-Pro-Phe, *p*-nitroanilide. Solvents and other chemicals were bought from either Fisher or Aldrich.

4.1. Stability studies in heterogeneous solution (> 70% DMF; ethanol studies)

Subtilisin was dissolved to a concentration of 10 mg/ml in 1 mM $CaCl_2$, and 50 μl of this solution was mixed into 2 ml of buffer: either 0.1 mM, 1 mM or 10 mM, to give a 10:1 or 100:1, or 1000:1 molar ratio of salt:enzyme, respectively. (In later studies, 50 μl of the enzyme stock was mixed with 200 μl of 100 mM buffer, instead of 2 ml of 10 mM buffer, and then lyophilized. No difference was ever observed between the preparations made by either method, but the latter method was more convenient.) The solution was frozen on dry ice and lyophilized to dryness and the resulting solid was placed in a siliconized 1 dram glass vial with a small teflon stir bar and covered with 3 ml of solvent under nitrogen. After 1–15 min of rapid stirring (30 Hz), the dispersion was fine enough to sample reproducibly. Time points were taken by removing 50 μl of the suspension with a wide bore pipet tip, then dissolving that in 450 μl 100 mM Tris buffer (pH 8). The sample was assayed immediately (although no change of activity was noticed after dissolution in buffer, even after several hours delay) by mixing 25 μl of the sample with 975 μl 0.5 mM sAAPFpNA in 100 mM Tris, pH 8, and monitoring the change in absorbance at 410 nm. All stability assays were stirred unless specifically mentioned.

4.2. Swirled assays

The enzyme was lyophilized as above and placed in siliconized 1 dram vial. A teflon stir bar was added and the enzyme solid was ground to a fine dust by rapid stirring in the absence of solvent. Three milliliters of DMF was then added and the enzyme was swirled using a wrist-action agitator (Thermolyne Maxi-Mix III) on a moderate setting.

4.3. Unstirred assays

One hundred microliters of the enzyme solution was aliquoted into each of 20 Eppendorf tubes, frozen on dry ice, and lyophilized to

dryness. One hundred microliters of DMF was added (under nitrogen) and the samples were left at room temperature until sampling. For each time point, 900 μl of 100 mM Tris buffer (pH 8) was added to each of two tubes, and the activity of the resulting solution was assayed immediately (as above).

4.4. Stability studies in homogeneous solution (≤ 70% DMF)

Water plus various percentages of cosolvent were mixed together and allowed to cool to room temperature. Thirty microliters of enzyme solution (30 mg/ml enzyme in 1 mM $CaCl_2$ + 1 M buffer) was added to 3.0 ml of DMF/water mixture. At various times, 50 μl samples were removed and added to 450 μl Tris buffer (pH 8.0) and assayed immediately (assay: 25 μl of enzyme sample was added to 975 μl 0.5 mM sAAPFpNA in 100 mM Tris, pH 8, and monitored at 410 nm).

4.5. Solubility studies

The enzyme was lyophilized and added to DMF + 1% water (as per the inhomogeneous solubility studies, above). At various intervals, 250 μl aliquots were removed, the solid was spun out (20 min. at 14,000 rpm in a microcentrifuge), and the sample was dialyzed (in a microdialyzer) to remove salts/DMF which might interfere with the Coomassie reagent. The samples were then assayed for protein content using the Coomassie Plus protein assay reagent (Pierce).

References

[1] G.A. Homandberg, J.A. Mattis, M. Laskowski, Biochemistry 17 (1978) 5220–5227.
[2] V. Schellenberger, H.-D. Jakubke, Angew. Chem., Int. Ed. Engl. 30 (1991) 1437–1449.
[3] K.L. Witte, P. Sears, R. Martin, C.-H. Wong, J. Am. Chem. Soc. 119 (1997) 2114–2118.
[4] C.-H. Wong, M. Schuster, P. Wang, P. Sears, J. Am. Chem. Soc. 115 (1993) 5893–5901.
[5] S. Riva, J. Chopineau, A.P.G. Kieboom, A.M. Klibanov, J. Am. Chem. Soc. 110 (1988) 584–589.
[6] W.J. Hennen, H.M. Sweeres, Y.-F. Wang, C.-H. Wong, J. Org. Chem. 53 (1988) 4939–4945.
[7] F. Effenberger, B.W. Graef, S. Osswald, Tetrahedron Asymmetry 8 (1997) 2749–2755.
[8] S.-C. Chen, S.-H. Wu, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc. 109 (1987) 2812–2817.
[9] J.B. Jones, J.F. Beck, Tech. Chem. (N.Y.) 10 (1976) 107–401.
[10] C.S. Chen, C.J. Sih, Angew. Chem. 101 (1989) 711–724.
[11] H.G. Park, H.N. Chang, J.S. Dordick, Biotechnol. Lett. 17 (1995) 1085–1090.
[12] Z. Gunata, M.J. Vallier, J.C. Sapis, R. Baumes, C. Bayonove, Enzyme Microb. Technol. 16 (1994) 1055–1058.
[13] P. Finch, J.H. Yoon, Carbohydr. Res. 303 (1997) 339–345.
[14] K. Chen, F.H. Arnold, Biotechnology 9 (1991) 1073–1076.
[15] P. Sears, C.-H. Wong, Biotechnol. Prog. 12 (1996) 423–433.
[16] Y.L. Khmelnitsky, S.H. Welch, D.S. Clark, J.S. Dordick, J. Am. Chem. Soc. 116 (1994) 2647–2648.
[17] K. Dabulis, A.M. Klibanov, Biotechnol. Bioeng. 41 (1993) 566–571.
[18] J.S. Dordick, Enzyme Microb. Technol. 11 (1989) 194–211.
[19] R. Affleck, X.-F. Xu, V. Suzawa, K. Focht, D.S. Clark, J.S. Dordick, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 1100–1104.
[20] P. Sears, M. Schuster, P. Wang, K.L. Witte, C.-H. Wong, J. Am. Chem. Soc. 116 (1994) 6521–6530.
[21] E. Zacharis, I.C. Omar, J. Partridge, D.A. Robb, P.J. Halling, Biotechnol. Bioeng. 55 (1997) 367–374.
[22] A. Zaks, A.M. Klibanov, Biochemistry 82 (1985) 3192–3196.
[23] Y. Yang, D. Zacherl, A.J. Russell, J. Am. Chem. Soc. 115 (1993) 12251–12257.
[24] S. Shubhada, P.V. Sundaram, Enzyme Microb. Technol. 17 (1995) 330–335.
[25] M.G. Kim, S.B. Lee, J. Mol. Catal. B 2 (1996) 127–140.
[26] C.R. Wescott, A.M. Klibanov, J. Am. Chem. Soc. 115 (1993) 1629.