Practical route to high activity enzyme preparations for synthesis in organic media

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A single pot method to rapidly prepare immobilised subtilisin Carlsberg and α-chymotrypsin gives 1000-fold greater catalytic activities in polar organic solvents than freeze-dried powders.

The application and utility of enzymes such as serine proteases and esterases in organic synthesis is well established. This has led to increased demand for enzyme preparations which exhibit high activity and reproducible selectivity, particularly in organic solvents.[1–4] The commonly used freeze-dried powders are known to exhibit low activity,[5,6] but are often used because they can be obtained in one step from commercially supplied enzymes. Enzymes immobilised onto a support often show higher activity in organic media but the types of support material reported may not always be readily available and well controlled immoblisation procedures are often required.[7–11] Recently, commercial cross-linked enzyme crystals (CLECs) have been introduced and these exhibit high activity, good stability and excellent reproducibility but are only currently available for certain enzymes.[12]‡ Here we demonstrate how enzyme preparations of very high activity can be rapidly and economically produced in a normal chemistry laboratory utilising a novel dehydration process.

The procedure employed is summarised in Fig. 1. The enzyme is first immobilised onto a support and for convenience we have used adsorption onto a standard silica chromatography gel.[13,14]§ For the enzymes tested the adsorption takes place quantitatively on stirring the protein with the silica in aqueous buffer solution at pH 7.8.¶ The immobilisation process disperses the biocatalyst over a large surface area and ensures good active site accessibility is obtained when the preparation is transferred to an organic solvent.‖ Following adsorption most of the aqueous solution is decanted from the silica–enzyme preparation ensuring the solid remains wetted. The key dehydration step is then achieved simply by rinsing the silica–enzyme preparation with successive aliquots of dry *n*-propanol, again keeping the solid wetted with solvent at all times. Where required, a final rinse with the reaction solvent of choice can be used to remove the propanol before the reactants are added. The propanol rinse rapidly removes water associated with the protein by a mechanism which minimises denaturation and appears to leave the majority of enzyme molecules in an active conformation. Ethanol and longer chain alcohols can also be used for the rinse, but methanol produces a much less active preparation.

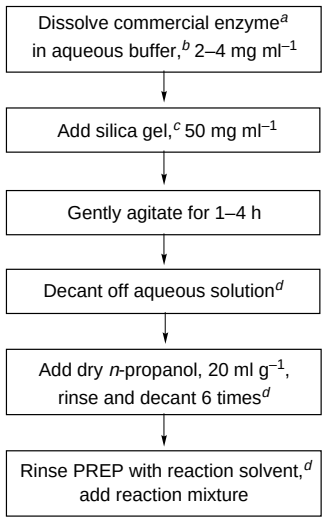

Fig. 1 Typical preparation of enzyme for use in organic solvent. [a] Highest purity available. [b] pH of maximum activity. [c] No special treatment required. [d] Ensure silica remains immersed.

The propanol rinsed enzyme preparations (PREPs) of subtilisin Carlsberg and α-chymotrypsin are found to exhibit very high activities in synthetically useful polar organic solvents such as acetonitrile (ACN) and THF.** Table 1 shows a comparison of the initial reaction rates obtained for a standard transesterification reaction in ACN using subtilisin Carlsberg prepared in different forms. At water levels of 1% (v/v) and above, the rates for the PREP are found to be comparable to the CLEC, and over 1000 times greater than the commonly used freeze-dried powders. Cross-linked enzyme crystals of chymotrypsin are not commercially available but under the same reaction conditions described in Table 1 the PREP of this enzyme exhibited transesterification activities of 21 and 131 nmol mg^{-1} min^{-1} in ACN, containing 1 and 4.5% v/v water, respectively. Again this is two orders of magnitude better than the freeze-dried powder.

If the PREP is removed from propanol and dried in air prior to assaying in the organic solvent most of the activity is lost and the residual level approaches that obtained for the lyophilised powder (see Table 1). However, aqueous suspensions of the silica adsorbed enzymes can be stored in the fridge for at least

Table 1 Effect of preparation type on subtilisin Carlsberg activity. Reaction conditions: 10 mM *N*-acetyl-L-tyrosine ethyl ester, 1 M propan-1-ol, in ACN containing water added as shown (% v/v). The mixture was incubated at 24 °C with constant reciprocal shaking (150 min^{-1}). Samples were analysed by HPLC on a Gilson 715 equipped with an ODS2 reversed phase column (Hichrom)

	Rate[a]/nmol mg^{-1} min^{-1}		
Enzyme form	0% H_2O v/v in ACN[b] (a_w < 0.01)	1% H_2O v/v in ACN (a_w = 0.22)	4.5% H_2O v/v in ACN (a_w = 0.55)
Freeze-dried powder	< 0.01	0.13	0.28
Cross-linked crystal (CLEC)	226	172	105
Propanol rinsed (PREP)	0.82	142	124
Propanol rinsed, air dried[c]	—	0.60	—

[a] mg refers to weight of enzyme in preparation. [b] Water content of dry ACN was < 0.007% v/v. [c] Sample prepared as in Fig. 1, placed in sealed jar over molecular sieves for 3 days, then equilibrated for a further 3 days over H_2O-saturated potassium acetate (a_w = 0.22).

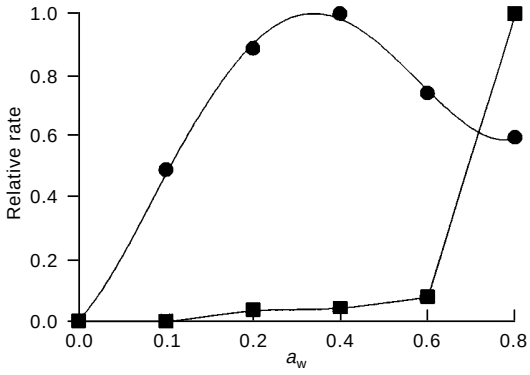

Fig. 2 Relative rate as a function of a_w for the transesterification reaction catalysed by subtilisin PREP (●) and freeze dried subtilisin Carlsberg (■). Rates for each preparation were normalised relative to the maximum value. These were 159.7 nmol mg^{-1} min^{-1} at a_w of 0.44 for the PREP and 3.3 nmol mg^{-1} min^{-1} at a_w of 0.76 for the freeze-dried powder. Reaction conditions as for Table 1.

3 weeks with negligible loss of activity and converted to the PREP as required.

It is perhaps surprising the method described here for preparing biocatalysts for reactions in low water media has not been reported previously. However, until recently the large hydration hysteresis effects obtainable with enzymes had not been fully recognised.[15,16] This work and that previously with CLECs[15] has shown that different methods of water removal can dramatically affect the enzyme activity obtained. Two possible explanations are (*i*) different dehydration protocols effect the amount of water left bound to the enzyme, or (*ii*) the conformation of the dried enzyme is very sensitive to the method of water removal. Most probably these two effects are intimately related.

According to previous studies the amount of water bound to a protein in solvent would be expected to be controlled by the thermodynamic water activity, a_w.[17,18] We therefore compared the catalytic activities of a freeze-dried powder and a PREP of subtilisin as a function of this parameter. Under these conditions differences in the amount of residual bound water should be eliminated and hence similar rate *vs*. a_w profiles might be expected for the PREP and powder. As can be seen in Fig. 2 this is not the case. The activity of the lyophilised powder continues to increase even up to a_w 0.76 while a maximal but much higher rate is obtained with the subtilisin PREP at a_w 0.44.†† Note that since the activity per unit weight of enzyme is much lower in the lyophilised powder (see Table 1), the rate profiles for the two preparations compared in Fig. 2 are relative rates normalised to the maximum value.

The different rate profiles could arise because the two forms of the enzyme differ in either water binding or the water required for catalytic activity. A large difference in water binding isotherms is unlikely, but kinetic factors may be significant. The water content of the lyophilised powder will be determined by its adsorption isotherm. In contrast, the treatment of the PREP imposes a water desorption process, for which the isotherm will be different due to hysteresis, and even the apparent equilibrium value may not be reached in the time allowed. An alternative hypothesis is that the PREP requires less water because the propanol dehydration process leaves a large proportion of the enzyme molecules in a conformation close to the active form. Only low levels of water may then be needed to promote catalysis and further increases in water availability provide no beneficial effect. With the dry freeze-dried powder the very low rates suggest most of the enzyme is initially inactive. In this case a water catalysed reorganisation process is probably required to convert the enzyme back to an active state since much greater water levels are needed to obtain high activity. The difference in profiles in Fig. 2 therefore reflects the fact that water plays different roles in promoting enzyme catalysis depending on the hydration history of the system.

We have shown here that very high activity propanol rinsed enzyme preparations (PREPs) can be obtained with both subtilisin Carlsberg and α-chymotrypsin, enzymes with very different secondary and tertiary protein structures. This suggests the procedure may find widespread application as a simple and economical way of preparing biocatalysts for reactions in organic media.

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Notes and References

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‡ Other enzyme forms recently reported to exhibit high activity in organic media include enzymes entrapped in sol-gels[19] and dissolved enzymes.[20]

§ Silica gel was Sigma S-0507, 230–400 mesh, 60 Å pores.

¶ The adsorption pH used corresponds to the optimum activity of the enzymes in aqueous solution. Typical adsorption conditions used; 20 ml of subtilisin Carlsberg (2 mg ml^{-1}) in 20 mM sodium phosphate buffer, pH 7.8, was mixed with 1 g of silica gel and the mixture shaken for 1 h at 25 °C. 20 ml of α-chymotrypsin (4 mg ml^{-1}) in 25 mM tris buffer, pH 7.8, containing 10 mM calcium chloride (to minimise autolysis) was shaken for 4 h at 25 °C. After mixing the adsorbed enzymes were stored in aqueous buffer at 4 °C.

‖ Since enzymes are generally insoluble in most organic solvents a heterogeneous reaction mixture is obtained and diffusion limitations can become important.

** It is often useful to carry out reactions at known thermodynamic water activity, a_w.[18] In polar solvents, in order to achieve a fixed a_w, a known concentration of water can be conveniently added to the system.[21] For ACN, a_w values of 0.11, 0.22, 0.44, 0.55 and 0.76 can be achieved by adding 0.5, 1.0, 2.7, 4.5 and 13% water (v/v) to the solvent.

†† Similarly, for freeze-dried chymotrypsin the rate increases with a_w, whereas an optimum rate is achieved at a_w of 0.55 for the PREP of this enzyme. The shift in rate *vs*. a_w for the chymotrypsin PREP in comparison to subtilisin PREP is interesting. This is also apparent when comparing profiles for the lyophilised powders of the two enzymes.

1 C. R. Wescott and A. M. Klibanov, *Biochim. Biophys. Acta*, 1994, **1206**, 1.
2 J. Broos, J. F. J. Engbersen, I. K. Sakodinskaya, W. Verboom and D. N. Reinhoudt, *J. Chem. Soc., Perkin Trans.* 1, 1995, 2899.
3 J. O. Rich and J. S Dordick, *J. Am. Chem. Soc.*, 1997, **119**, 3245.
4 G. A. Hutcheon, M. C. Parker, A. James and B. D. Moore, *Chem. Commun.*, 1997, 931.
5 K. Dabulis and A. M. Klibanov, *Biotechol. Bioeng.*, 1993, **41**, 566.
6 O. Almarsson and A. M. Klibanov, *Biotechnol. Bioeng.*, 1996, **49**, 87.
7 A. O. Triantafyllou, D. B. Wang, E. Wehtje and P. Adlercreutz, *Biocat. Biotrans.*, 1997, **15**, 185.
8 Z. Yang, A. J. Mesiano, S. Venkatasubramanian, S. H. Gross, J. M. Harris and A. J. Russell, *J. Am. Chem. Soc.*, 1995, **117**, 4843.
9 E. Wehtje, P. Adlercreutz and B. Mattiasson, *Biotechnol. Bioeng.*, 1993, **41**,171.
10 J. F. Diaz and K. J. Balkus, *J. Mol. Cat.*, 1996, **2**, 115.
11 M. T. Reetz, A. Zonta, A. and J. Simpelkamp, *Biotechnol. Bioeng.*, 1996, **49**, 527.
12 Y. F. Wang, K. Yakovlevsky and A. L. Margolin, *Tetrahedron Lett.*, 1996, **37**, 5317.
13 T. Zoungrana, G. H. Findenegg and W. Norde, *J. Colloid Interface Sci.* 1997, **190**, 437.
14 V. M. Stepanov, E. Y. Terenteva, T. L. Voyushina and M.Y. Gololobov, *Bioorg. Med. Chem.*, 1995, **3**, 479.
15 J. Partridge, G. A. Hutcheon, B. D. Moore and P. J. Halling, *J. Am. Chem. Soc.*, 1996, **118**, 12 873.
16 F. X. Yang and A. J. Russell, *Biotechnol Bioeng.*, 1996, **49**, 700.
17 G. A. Hutcheon, P. J. Halling and B. D. Moore, *Methods Enzymol.*, 1997, **286**, 465.
18 G. Bell, P. J. Halling, B. D. Moore, J. Partridge and D. G. Rees, *Trends Biotechnol.*, 1995, **13**, 468.
19 S. Sato, T. Murakata, M. Ochifuji, M. Fukushima and T. Suzuki, *J. Chem. Eng. Jpn.*, 1994, **27**, 732.
20 V. M. Paradkar and J. S. Dordick, *J. Am. Chem. Soc.*, 1994, **116**, 5009.
21 G. Bell, A. E. M. Janssen and P. J. Halling, *Enzyme Microb. Technol.*, 1997, **20**, 471.

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