CHEMCOMM Communication
www.rsc.org/chemcomm

Enzyme-coated micro-crystals: a 1-step method for high activity biocatalyst preparation

Michaela Kreiner,[a] Barry D. Moore,[b] and Marie Claire Parker*[a]

[a] *Department of Chemistry, University of Glasgow, Joseph Black Building, Glasgow, UK G12 8QQ. E-mail: mariec@chem.gla.ac.uk*
[b] *Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, UK G1 1XL*

Received (in Cambridge, UK) 19th January 2001, Accepted 4th May 2001
First published as an Advance Article on the web 25th May 2001

A rapid, inexpensive method for producing water-soluble enzyme-coated micro-crystals which exhibit dramatically enhanced catalytic activity and stability in non-aqueous media and can be re-dissolved easily in aqueous solution is described.

Enzymes can frequently show poor activity in organic media when compared to their respective activities in aqueous solution.[1] It has been well documented that lyophilisation (a frequent choice of enzyme preparation for storage and use in organic media) causes pronounced structural perturbation for most proteins, including one of the enzymes in this study, subtilisin Carlsberg (SC).[2,3] To overcome these problems, many strategies aimed at optimising enzyme activity, such as a range of immobilisation methods and manipulation of the micro-environment have been studied. Amongst others, the mode of enzyme preparation,[4–6] co-lyophilisation with lyoprotectants[7] and salts,[8,9] imprinting with substrates and substrate analogues,[10] additives[11] and cross-linked protein crystals[12] have been explored. Here we demonstrate how enzyme preparations of high activity can be rapidly and economically produced using a novel process[13] resulting in the formation of protein-coated micro-crystals (PCMCs).

The procedure for the preparation of PCMCs is summarised in Fig. 1. An aqueous protein solution is mixed with a concentrated solution of an excipient, such as a salt (*e.g.* K_2SO_4), a sugar or an amino acid. A primary requirement is that the excipient (the crystal-forming component) should show high solubility in water and negligible solubility in the precipitating water-miscible organic solvent. This combined aqueous mixture is then added dropwise with rapid mixing to a water miscible solvent (*e.g.* 1-PrOH), whereupon the protein and excipient instantly co-precipitate. The structure of the co-precipitate (SC loading in SC–K_2SO_4 = 8 wt%) typically consists of micron-sized crystals with protein molecules located at their surface. A major advantage is that during the process the organic solvent dehydrates the enzymes by a mechanism that minimises denaturation and appears to leave the majority of enzyme molecules in an active conformation. In addition a fine-particle (0.1–5 μm) suspension is formed so that the protein-coated crystals are homogeneously dispersed in the organic solvent. We have found that routine transferral of this suspension either by pipette or an automated liquid-handling system is very straightforward. This therefore means that enzyme-coated micro-crystals are ideally suited for use in biocatalyst screening programmes. For use in organic solvents, the enzyme-coated micro-crystals can be dried *in situ* to form a fine powder, or the precipitating solvent can be decanted off and replaced by a different solvent. Alternatively for aqueous applications, the enzyme-coated micro-crystals can be rapidly re-dissolved into aqueous solution.†

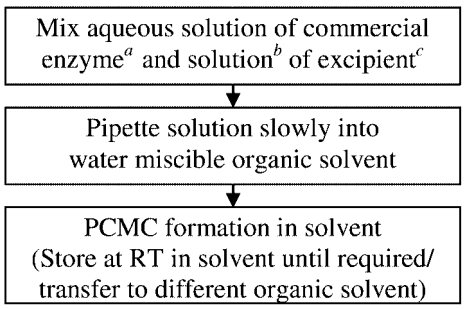

Fig. 1 Procedure for preparation of protein-coated micro-crystals.[a] At pH optimum, both pure and impure preparations can be used. [b] At or near saturation. [c] Component of crystal immobilisation matrix consists of either a salt, an amino acid or a sugar.

Table 1 shows a comparison of the catalytic activity of SC either as a lyophilised powder or after formation of the SC-coated K_2SO_4 micro-crystals (SC-K_2SO_4)‡ in different solvents. The transesterification reaction of *N*-AcTyrOEt using 1-PrOH was monitored in acetonitrile (AcCN–1% H_2O v/v). Significantly, the catalytic rate for all PCMC preparations was over three orders of magnitude higher than that typically found using a lyophilised preparation.[5] Among the solvents tested (Table 1), propan-1-ol and ethanol were found to be the most effective, whereas with SC polar aprotic solvents gave lower activity, but nevertheless markedly increased the activity compared to lyophilised preparations. We found that the water content of the organic solvent used for precipitation is quite flexible (0–20% H_2O v/v). Only at water contents greater than 20% is the activity significantly reduced.

Transmission electron microscopy (TEM) showed that co-precipitates formed in organic solvent were crystalline, resulting in rectangular crystals as shown in Fig. 2 for SC–K_2SO_4 produced in 1-PrOH. Tapping-mode atomic force microscopy (TM-AFM) imaging (data not shown) shows that the protein molecules are located in a fairly uniform layer on the surface of the crystal, where they are easily accessible to the substrate. Interestingly K_2SO_4 crystals formed in the absence of protein are larger. This too indicates that enzyme molecules, located at

Table 1 Effect of preparation method on the transesterification performance in AcCN–1% H_2O (v/v) of PCMC-SC

Enzyme form	Precipitating solvent	Relative rate[a]
Freeze-dried[b]	—	0.1
PCMC	1-PrOH–1% H_2O	97
PCMC	1-PrOH–7% H_2O	90
PCMC	1-PrOH–25% H_2O	79
PCMC	EtOH[c]	100
PCMC	AcCN[c]	45
PCMC	Acetone[c]	52
PCMC	THF[c]	76
PCMC, after storage (14 months, RT)	1-PrOH [c]	86

[a] Relative rate (100 = 29% conversion measured after 3 h). Reaction conditions: 10 mM *N*-acetyl-L-tyrosine ethyl ester, 1 M 1-PrOH, 0.47 mg SC in 3 ml of AcCN–1% H_2O (v/v), T = 25 °C and shaken at 200 rpm. [b] Freeze-dried (10 mM Tris-buffer, pH = 7.8). [c] Containing 1% (v/v) water. Samples were analysed by HPLC.¶

 DOI: 10.1039/b100722j

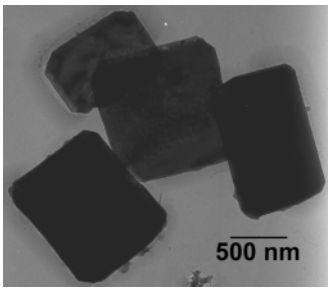

Fig. 2 Transmission electron microscopy (TEM)‖ image of K_2SO_4 crystals co-precipitated with SC using 1-PrOH–1% H_2O as precipitating solvent.

the crystal surface, limit crystal growth by acting like a 'poison'.

There are two ways the PCMCs can be stored, as a dry powder or *in situ* as a suspension in 1-PrOH. Historically, drying to a powder has been the preferred route for storage of enzyme and protein powders, but we find a loss of (up to 40%) catalytic activity as a result of air-drying, which can be regained by coating in surfactant (PPG 2025 or Span 80). Alternatively, when stored directly in the precipitating solvent, remarkably a suspension of PCMC-subtilisin can be stored for 14 months at RT with negligible loss in activity (see Table 1).

In order to assess the generality of this novel method we extended our study to lipases employing the kinetic resolution of (±)-1-phenylethanol as a model reaction. We chose as a starting point a lipase screening kit consisting of a set of 10 lipases which are available in lyophilised form and are of varying purity. Initially we screened the lipases in the set for high enantioselectivity E[14] (E > 200) in a model reaction: the conversion of (*R*,*S*)-phenylethanol to (*R*)-phenyl ethyl acetate using vinyl acetate as acyl donor. The activities of these lipases either *as received* or after precipitation to form the enzyme-coated micro-crystals are shown in Fig. 3. Remarkably, the catalytic activity is enhanced significantly for most of the enzyme-coated lipases§ when compared to the *as received* preparations. For PCMC-*Pseudomonas* sp. lipase and PCMC-using lipase B from *Candida antarctica* (CALB) the reaction is complete in 0.5 and 2 h, respectively. PCMC-*Pseudomonas* sp. lipase was found to be 200-fold more active than the *as received* preparation. Coated micro-crystals of CALB and lipase from *M. miehei* showed a 5 and 15-fold increase in catalytic activity, respectively. No change in the enantiomeric ratio (E > 200 in all cases) was observed. A 60-fold increase in the activity of K_2SO_4–CALA (E = 1) was observed. However, one enzyme system K_2SO_4–*Alcaligenes sp.* showed no improvement.

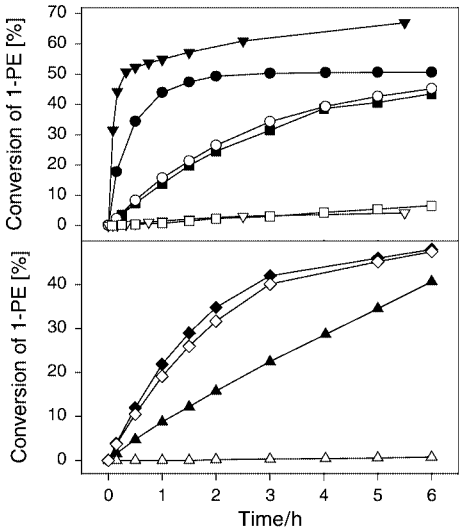

Fig. 3 Comparison in catalytic activity of lipase-coated micro-crystals and the *as received* preparations for the kinetic resolution of (±)-1-phenyl-ethanol (1-PE). PCMCs were made using K_2SO_4 as support and 1-PrOH–1% H_2O as precipitating solvent. (▼) PCMC-*Pseudomonas* sp. lipase; (▽) lipase from *Pseudomonas* sp.; (●) PCMC-CALB; (○) CALB; (■) PCMC-*Mucor miehei* lipase; (□) lipase from *Mucor miehei* lipase; (◆) PCMC-*Alcaligenes* sp. lipase; (◇) *Alcaligenes* sp. lipase; (▲) PCMC-CALA; (△) CALA. Reaction conditions: dry *tert*-butyl methyl ether (2.5 ml), lipase** (2.5 mg, except for CALA: 5 mg), 1-PE (0.1 M), vinyl acetate (0.3 M), T = 30 °C and shaken at 200 rpm. Samples were analysed by HPLC.††

The preparation of enzyme-coated micro-crystals for routine biocatalysis in organic media is fast, cheap, and requires only standard laboratory equipment. Suspensions of highly active PCMCs show significantly improved ease of handling/dispensing and storage in liquids compared to their lyophilised counterparts. This suggests that the methodology may find widespread application as a generic method for the production of biocatalysts with predictable morphology and handling capacity in automated screening applications.

We would like to thank Dr David McComb and Mr Jim Gallagher for the use of EM facilities at the University of Glasgow. The work was supported by the BBSRC, a David Phillips Research Fellowship (MCP) and funding for MK. We would also like to thank Mr Andreas Elmecker for image processing.

Notes and references

† Specific activity of subtilisin Carlsberg, *Candida antarctica* lipase B and *Mucor miehei* lipase is fully retained upon re-dissolution into aqueous solution. Other enzymes used were not investigated in this regard.

‡ Subtilisin Carlsberg, Type VIII (Sigma, UK) (4 mg) was dissolved in 100 μl of Tris buffer (10 mM, pH 7.8). To this, 300 μl of a saturated solution of excipient was added. This combined solution was then added drop-wise to a shaking vial (150 rpm) containing 6 ml of the organic solvent.

§ PCMC-lipases: *Candida antarctica* lipases L-2, and L-5, *Pseudomonas* sp. lipase L-6, *Mucor miehei* lipase L-9, *Alcaligenes* sp. lipase L-10 (Roche Diagnostics, Germany). Procedure as for SC except: 2.5 mg of L-2, L-6, L-9, L-10; 5 mg of L-5 dissolved in 62 μl of phosphate buffer (50 mM, pH 7.0) and 188 μl of a saturated solution of K_2SO_4 was then added. Addition to 3.75 ml of 1-PrOH–1% H_2O as described above. The PCMCs were washed with dry *tert*-butyl methyl ether to remove 1-PrOH.

¶ WATERS 2690 HPLC (Waters, UK) equipped with a Spherisorb S5 ODS2 column (4.6 × 250 mm) (Waters, UK). λ_{max} = 280 nm.

‖ Air-dried PCMCs were examined using a Jeol JEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

** Weight of *as received* powders is the same as weight of powder used for formation of PCMCs. Protein loading not determined.

†† HPLC (as above) using a Chiracel OD column (250 × 4.6 mm) (Daicel, Japan). λ_{max} = 210 nm.

1 M. Klibanov, *Trends Biotechnol.*, 1997. **15**, 97.
2 K. Griebenow and A. M. Klibanov, *Biotechnol. Bioeng.*, 1997, **53**, 351.
3 K. Griebenow and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, 1995, **92**, 10969.
4 H. Noritomi, O. Almarsson, G. L. Barletta and A. M. Klibanov, *Biotechnol. Bioeng.*, 1996, **51**, 95.
5 J. Partridge, P. J. Halling and B. D. Moore, *Chem. Commun.*, 1998, **7**, 841.
6 T. Ke and A. M. Klibanov, *Biotechnol. Bioeng.*, 1998, **57**, 746.
7 K. Dabulis and A. M. Klibanov, *Biotechnol. Bioeng.*, 1993, **41**, 566.
8 Y. L. Khmelnitzky, S. H. Welch, D. S. Clark and J. S. Dordick, *J. Am. Chem. Soc.*, 1994, **116**, 2647.
9 M. T. Ru, J. S. Dordick, J. A. Reimer and D. S. Clark, *Biotechnol. Bioeng.*, 1999, **63**, 233.
10 J. O. Rich and J. S. Dordick, *J. Am. Chem. Soc.*, 1997, **119**, 3245.
11 F. Theil, *Tetrahedron*, 2000, **56**, 2905.
12 J. J. Lalonde, C. Govardhan, C. Khalaf, A. G. Martinez, K. Visuri and A. Margolin. *J. Am. Chem. Soc.* , 1995, **117**, 6845.
13 UK Patent disclosed: WO 00/69877, *Rapid dehydration of proteins*, M. C. Parker, B. D. Moore, P. J. Halling and J. Partridge.
14 C-S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294.