Solubilisation of α-chymotrypsin by hydrophobic ion pairing in fluorous systems and supercritical carbon dioxide and demonstration of efficient enzyme recycling

Karima Benaissi,[a,b] Martyn Poliakoff*[a] and Neil R. Thomas*[b]

Received 10th March 2009, Accepted 21st August 2009
First published as an Advance Article on the web 29th September 2009
DOI: 10.1039/b904761a

Hydrophobic ion-pairing (HIP) with the fluorinated surfactant KDP 4606 (KDP) was used to extract the protein α-chymotrypsin (CMT) into perfluoromethylcyclohexane (PFMC). The diameter of the solubilised CMT-KDP complexes formed in PFMC was determined by dynamic light scattering (DLS) to be 25 nm which suggested the formation of a protein aggregate containing ~100 protein molecules surrounded by KDP 4606 surfactant molecules per particle. The catalytic activity of the protease CMT either solubilised by HIP or as the suspended native enzyme has been investigated in both a fluorous biphasic system (FBS) and a supercritical carbon dioxide ($scCO_2$) batch reactor. Transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with *n*-butanol or *rac*-2-butanol was catalysed by the protease in the FBS hexane-PFMC or $scCO_2$ at 40 °C. Under comparable conditions, the amount of transesterification of the solubilised protease–surfactant (CMT-KDP) complex in PFMC (6–10%) was shown to be significantly higher than that of the suspended protease (1–3%) in either hexane–PFMC or $scCO_2$. This suggested the formation of a catalytically active CMT-KDP aggregate in PFMC. The CMT-KDP complex which is retained in the fluorous phase on cooling the solution was successfully reused over four cycles with no loss of activity.

Introduction

The lipids, esters, acids and alcohols that are widely used as starting materials for the commercial production of important pharmaceuticals, cosmetics and food products, are often poorly soluble in water. Therefore, biocatalysis in non-aqueous media to produce these materials has attracted considerable attention in recent decades.[1] Given the move to replace hydrocarbon solvents with more environmentally benign alternatives, one area of significant research activity is the identification of new solvents and reaction systems that are compatible with the use of enzymes.[1]

Different strategies, such as the suspension of the enzyme in an organic solvent,[2] lipid-coated enzymes,[3] microencapsulation of enzymes in reverse-micelles,[4] immobilisation of enzymes on a microgel-matrices or within membranes,[5] covalent attachment of polymers (pegylation) to the enzyme,[6] stabilisation of enzymes forming cross-linked enzyme crystals (CLECs®)[7] or cross-linked enzyme aggregates (CLEAs®),[7,8] aimed at increasing the stability and active site accessibility of enzymes have been employed to enhance enzymatic activity in organic media. However, the effect on the catalytic activity of these modified enzyme forms is unpredictable and modifications can result in a considerable loss of enzymatic activity.[3,4,6–11]

The technique of hydrophobic ion-pairing (HIP) has previously been reported to solubilise proteins in hydrocarbon solvents by forming ion pairs with anionic organic surfactants such as Aerosol-OT (AOT) (see Fig. 1 for structure).[12] In some cases, including with protease α-chymotrypsin (CMT),[1,13–16] an increase in catalytic activity was observed with these anionic surfactant–enzyme complexes in the organic solvent.[1,13–19] The use of HIP to solubilise enzymes using anionic fluorinated surfactants such as KDP 4606 (see Fig. 1 for structure) and Krytox 157 FSL developed from these studies.[10,13,19] In the case of CMT, the enzyme–CO_2-philic surfactant complex formed has been shown to be active and dispersible in CO_2.[10,13]

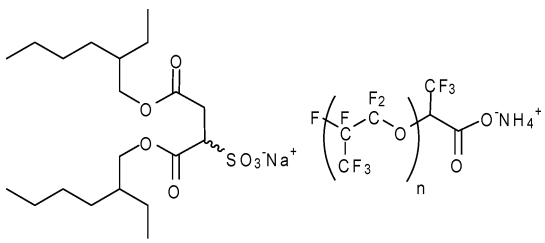


Fig. 1 Structures of the anionic surfactants sodium *bis*(2-ethylhexyl) sulfosuccinate (Aerosol-OT, AOT) and KDP 4606 (DuPont®).

In this paper we describe the use of both a fluorous biphasic system (FBS) and supercritical carbon dioxide ($scCO_2$) as solvents for a transesterification catalysed by CMT.

[a]*Clean Technology Group, School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom. E-mail: martyn.poliakoff@nottingham.ac.uk*
[b]*Centre for Biomolecular Sciences, School of Chemistry, University of Nottingham, University Park, Nottingham, NG7 2RD, United Kingdom. E-mail: neil.thomas@nottingham.ac.uk*

Only a few examples of biocatalysis under FBS conditions have been reported.[9,10,13] *Candida rugosa* lipase (CRL) suspended in hexane was used to catalyse the separation of *rac*-2-methylpentanoic acid *via* fluorinated tagging of one of the two enantiomers using a highly fluorinated alcohol.[9] This promising approach was shown to allow easy separation and recovery of both product and catalyst. This approach was recently developed further through the use of enzymes solubilised in fluorous solvents by HIP to allow a homogeneous reaction to be achieved in a fluorous biphasic system,[10] the enzyme was retained in the fluorous phase allowing easy recycling.

This paper reports new data on the solubilisation of CMT using HIP and describes efficient recycling of the HIP solubilised protease for transesterification reactions catalysed in both a FBS and $scCO_2$.

Materials and methods

α-Chymotrypsin (CMT, type II from bovine pancreas, 51 U mg^{-1}) was obtained from Aldrich. Chemicals: *N*-acetyl-L-phenyl alanine ethyl ester (APEE), *n*-butanol and *rac*-2-butanol were purchased from Aldrich. The organic surfactant sodium *bis*(2-ethyl-2-hexyl)succinate (AOT) was purchased from Fluka and the fluorinated surfactant KDP 4606 was kindly donated by DuPont®. Perfluoromethylcyclohexane (PFMC) was purchased from Fluorochem. All organic solvents were purchased at the highest purity available from Aldrich. All commercially available reagents and solvents were used without further purification. High purity CO_2 was purchased from CryoService. Gases hydrogen, helium and air were purchased from BOC Gases.

Safety note

Experiments with $scCO_2$ involve high pressures and should only be carried out in equipment with the appropriate pressure rating and safety operating procedures.

Solubilisation of CMT-KDP in PFMC

The preparation of protein–surfactant complexes has been described previously.[13] CMT (51 U, 0.02 mM) dissolved in sodium phosphate buffer (1 mL, 10 mM, pH 6.9), 5 mM $CaCl_2$ (1 mL) was stirred with AOT (2.3 mM) in hexane (1 mL) or KDP (0.7 mM) in PFMC (1 mL) at room temperature at 500 rpm for 10 min and centrifuged at 8000 rpm (4600*g*) for 2 min to allow phase separation to occur. The lower fluorous phase was then collected and diluted ten-fold in PFMC (1 mL) and the UV absorbance of the protein was measured at 280 nm (value[20,21] of $\varepsilon_{240} = 50\,585\ M^{-1}\ cm^{-1}$) using an Agilent 8453 UV spectrophotometer. Comparable experiments were set up using AOT (1 mg mL^{-1}, 2.3 mM) in hexane (1 mL) and the amount of CMT-AOT in hexane was determined by UV spectroscopy. All experiments were performed in triplicate.

Determination of the critical micellar concentration (CMC) of KDP 4606 in PFMC

The method used to determine the critical micellar concentration (CMC) of the fluorinated surfactant by UV-visible spectroscopy has been described previously.[22] Solutions of KDP in the range 5 mM to 70 mM in PFMC (1 mL) were stirred with 1 ml of sodium phosphate buffer (10 mM, pH 6.9) or sodium acetate buffer (10 mM, pH 3.8) at 500 rpm for 10 min and centrifuged at 8000 rpm (4600*g*) for two min to allow phase separation to occur. The lower fluorous phase was then collected and the concentration of surfactant analyzed by measuring the absorbance at 240 nm ($\varepsilon_{240} = 59.4\ M^{-1}\ cm$),[10] as described above. This measurement was repeated in triplicate.

Dynamic light scattering (DLS)

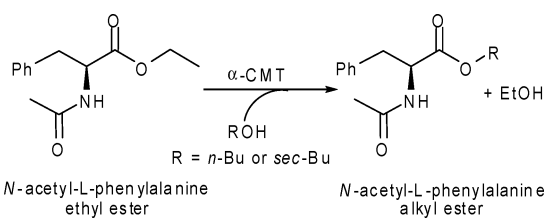
DLS measurements were carried out at 25 °C on a Zetasizer Nano S at Malvern Instruments. The particle size was taken as a mean value of three measurements. CMT dissolved in aqueous buffer; AOT and CMT-AOT dissolved in hexane; and CMT-KDP and KDP dissolved in PFMC were measured at 0.02 mM, 2.3 mM, 0.02 mM, 0.7 mM and 0.02 mM, respectively. The data was analyzed with the following fixed parameters:[23] refractive index 1.333, 1.372, 1.277, 1.39, 1.4 and 1.45 (water, hexane, PFMC, AOT, KDP and protein respectively) and viscosity (cP) 1.0019, 0.294 and 1.561 (water, hexane and PFMC respectively).

Estimation of water content using coulometric Karl Fisher titration

The water content of hexane, PFMC, CMT-AOT in hexane, and CMT-KDP in PFMC based on coulometric Karl Fisher titration was estimated using a Mitsubishi moisture meter CA-100 following the manufacture's method. Samples (2 to 10 mg) were added into the titration solution using a syringe and the mass of sample introduced was indicated to the titrator. Titration with iodine was performed under stirring at 700 rpm. The percentage of water in the samples was measured in triplicate. The sensitivity of the Coulometric Karl Fisher titrator is 1 ppm water content.[24]

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with *n*-butanol or *rac*-2-butanol was catalysed by the protease α-chymotrypsin (CMT, MW = 51 000). The protein was used either in solubilised form as its HIP complex (CMT-AOT in hexane or CMT-KDP in PFMC) or as the suspended native form in hexane, hexane-PFMC (v/v 1 : 1) or $scCO_2$ (100 bar) at 40 °C for two hours. The reaction studied is shown in Scheme 1.



Scheme 1 Transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) (4.2 mM) with *n*-butanol or *rac*-2-butanol (0.45 M) was catalysed by α-chymotrypsin (CMT, 102 U, 0.04 mM) in hexane or hexane–PFMC v/v 1 : 1 (2 mL) or $scCO_2$ (100 bar, 8.5 mL) at 40 °C for two hours.

CMT (51 U, 1 mg) solubilised in PFMC (1 mL) was added to the substrates *N*-acetyl-L-phenylalanine ethyl ester (APEE) (8.5 μmol, 2 mg) and *n*-butanol (0.9 mmol, 80 μL) or *rac*-2-butanol (0.9 mmol, 80 μL) in hexane or hexane/PFMC v/v 1 : 1 (2 mL). A comparable reaction was set up in $scCO_2$ (100 bar, 8.5 mL) and the system was left stirring at 40 °C for two hours. When the reaction was finished, the system was cooled down to 0 °C in an ice bath to allow phase separation to occur. Aliquots of the upper organic phase were collected and analyzed as described above. A fresh batch of substrates was then added to the lower fluorous phase and the reaction was repeated as described above. The yields of products were determined using a Shimadzu GLC-2010 GLC equipped with an AOC 20i autosampler and fitted with a RTX5-FAST column (fused silica, crossbond 5% diphenyl–95% dimethyl polysiloxane, 10 m × 0.1 mm × 0.1 μm) from Restek. A temperature programme was used for each run; the oven temperature was increased from 100 °C to 250 °C at 25 °C per minute and held at 250 °C for two minutes. Samples of 1.0 μL were injected onto the column with a split ratio of 75 : 1. The injector temperature was 250 °C and the FID temperature was 350 °C.

Batch reactions

Enzymatic reactions in organic solvents and PFMC were carried out in 2 mL septum-sealed Supelco glass micro reaction vessels equipped with magnetic stirrer bars and stirred at 500 rpm in a silicon oil bath thermostated to the indicated temperature using a Yellowline MST basic hot plate equipped with a Yellowline TC1 thermostat.

Reactions in $scCO_2$ were performed in a stainless-steel high pressure batch reactor specially designed at the University of Nottingham.[25] The batch reactor (autoclave) equipped with a magnetic stirrer bar has an internal volume of 8.5 mL and was placed on an IKA Labotecknick RCT basic stirring plate. Solid substrates and reactants and stirrer bar were added into the reactor prior to sealing. Liquid substrates and reactants were added using a Gilson Pipetman micropipette into the autoclave then sealed using a safety valve. A band heater, thermocouple, input and output pressure pipes were then connected to the reactor to enable temperature and pressure control of the system. The system was then heated to the desired temperature and liquid CO_2 was pumped into the reactor using a high pressure NWA PM-101 Pickel pump until the desired pressure was achieved. The reaction mixture was stirred for the period of time specified and the system was then depressurised by placing the autoclave in a dry ice–acetone bath. The residual mixture in the autoclave was dissolved in acetone (3 mL) and centrifuged for 2 min. at 8000 rpm (4600*g*).

Aliquots (50 μL) were diluted in acetone (1 mL) and substrates and products were analyzed by gas-liquid chromatography (GLC) using a Shimadzu GC 2010 chromatograph equipped with a Shimadzu AOC-20Si autosampler using helium as the carrier gas. The structure of products was further identified using GLC and GLC-MS by comparing the retention times and fragmentation patterns with those of authentic samples. GLC-MS was performed using a ThermoFinnegan Polaris-Q trap GC-MS equipped with a DB-5 (30 × 0.25 × 0.25 μm film thickness) fused silica column. Helium was used as a carrier gas and sample ionisation was carried out using electron impact (EI) at 70 eV.

Table 1 Solubilisation of CMT-AOT in hexane and CMT-KDP in PFMC

	Complexation efficiency[a] (%)	
Concentration of $CaCl_2$ in buffer (mM)	CMT-AOT in hexane	CMT-KDP in PFMC
0	68 ± 3	29 ± 4
5	96 ± 3	51 ± 4

[a] Complexation efficiency (%) = [mass of protein solubilised in organic (or fluorous) phase]/[initial mass of protein] × 100

Results and discussion

Solubilisation of CMT-KDP in PFMC

There are suggestions in the literature that the presence of a salt, such as $CaCl_2$ in the aqueous buffer from which an enzyme is extracted strongly influences both the solubility and activity of the enzyme in the receiving organic solvent.[12,16,26] It has been postulated that at high salt concentrations, that the salt matrix may act as a rigid structure that would protect the enzyme from the organic solvent.[26] Also, the salt matrix, being highly polar, might help to maintain the native structure of the enzyme in organic media.[26]

CMT was therefore dissolved in a sodium phosphate buffer (10 mM, pH 6.9) with or without addition of 5 mM $CaCl_2$. The protein was then extracted into hexane using AOT or into PFMC using KDP and the complexation efficiency of CMT-AOT in hexane and CMT-KDP in PFMC were estimated by measuring the UV absorbance of the protein at 280 nm. The results obtained are summarised in Table 1.

Adding $CaCl_2$ to the aqueous buffer was observed to facilitate the extraction of the protein into the organic (or fluorous) phase since the complexation efficiency was observed to be increased by about 40–50% in both cases. Under comparable conditions, the amount of enzyme solubilised in hexane using AOT (96% of the starting material) was higher than that obtained using KDP in PFMC (51%).

The differences in complexation efficiencies observed between the two surfactants tested may be due to the structure of the surfactants (see Fig. 1). Parameters such as the size, polarity and hydrophobicity[12] of the fluorinated tail on the surfactant are likely to influence the contact between surfactant and protein.

Determination of the critical micelle concentration (CMC) of KDP 4606 in PFMC

By plotting absorbance against concentration of KDP[10] it was possible to determine the CMC of KDP in PFMC as 48 mM at room temperature (maximum on the graph shown in Fig. 2).

The concentration of KDP (0.7 mM) used to solubilise CMT in PFMC in our experiments is far below the measured CMC (48 mM) which suggests that the solubilisation process used does not lead to the formation of micelles.

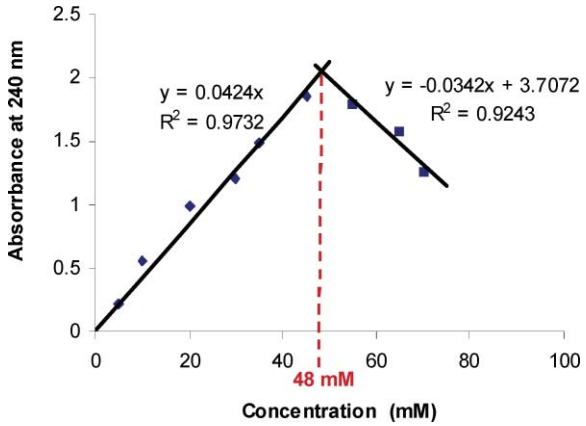


Fig. 2 UV absorbance *versus* concentration for KDP 4606 at 240 nm for the determination of the critical micelle concentration (CMC), indicated by dashed line.

Dynamic light scattering (DLS)

The particle size of CMT solubilised in phosphate buffer (10 mM, pH 6.9), CMT-AOT complexes in hexane and CMT-KDP complexes in PFMC were determined using DLS. The results are shown in Fig. 3.

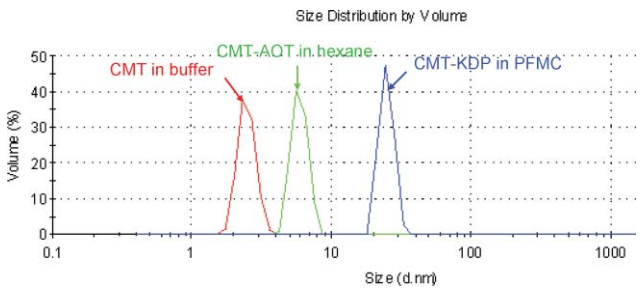


Fig. 3 DLS traces showing different particle sizes of CMT in different media (native CMT in buffer, CMT-AOT in hexane and CMT-KDP in PFMC), indicating that CMT-KDP is clustered under these conditions.

The particle diameter of CMT dissolved in aqueous buffer was determined to be 2.5 nm; consistent with the size of a single molecule of CMT. The diameter of CMT-AOT particles in hexane was found to be around 6.0 nm which is similar to the size of a single molecule of CMT (2.5 nm) plus two molecules of AOT (size of AOT 1.2 nm).[15] This suggests the presence of individual CMT particles surrounded by the surfactant AOT in hexane.

Particles of CMT-KDP in PFMC were found to have a diameter of 25 nm which would correspond to the formation of an aggregate of about 100 protein molecules surrounded by the surfactant KDP in PFMC. KDP is estimated to have a length of ~1.4 nm. The aggregation observed for the solubilised CMT in PFMC might be related to the very low water content of the hydrophobic fluorinated solvent.[27]

Estimation of water content using coulometric Karl Fisher titration

The water content of hexane; AOT in hexane and CMT-AOT in hexane; PFMC; KDP in PFMC; and CMT-KDP in PFMC were determined using the coulometric Karl Fisher titration.

Table 2 Water content of hexane, AOT and CMT-AOT in hexane, PFMC, KDP and CMT-KDP in PFMC estimated using coulometric Karl Fisher titration

Sample	Water content (ppm)
Hexane	34 ± 4
AOT in hexane	45 ± 5
CMT-AOT in hexane	41 ± 8
PFMC	<1[a]
KDP in PFMC	<1[a]
CMT-KDP in PFMC	<1[a]

[a] Sensitivity of the coulometric Karl Fisher Titrator 1 ppm.[24]

The experiments were performed in triplicate and the results obtained were reproducible as shown in Table 2.

Less than 50 ppm water was detected in a sample of CMT-AOT in hexane which is mainly due to the water content of hexane. The amount of water in the PFMC samples was below the detection limit of the instrument (1 ppm).[24] To ensure that the fluorinated solvent PFMC was not interfering with the detection of Karl Fisher titration, known quantities of water were purposely added to PFMC and the water content was measured using Karl Fisher titration. In these cases, the amount of water added was accurately detected by the machine.

The very low amount of water detected for CMT-KDP in PFMC may explain the formation of protein aggregates observed by DLS (Fig. 3). In a recent paper, Akbar *et al.* describe the solubilisation of subtilsin Carlsberg and CALB in isooctane with or without extraction from an aqueous buffer solution.[27] They observed that the proteins directly solubilised in a solution of AOT in isooctane containing 0.2% of water would form clusters/aggregates in the organic solvent. However, the enzymes dissolved by extraction from an aqueous buffer solution would appear as single proteins surrounded by the surfactant in isooctane.

These results are in agreement with our own observation that proteins tend to form aggregates when the amount of water is limited in their environment, such as the in very hydrophobic fluorous solvent PFMC. Akbar *et al.* suggested that this aggregation might provide physical protection of the enzymes, thus maintaining them in an active form and able to catalyse the transesterification of a natural product with vinyl butyrate in different organic solvents. In their case, the enzyme aggregates seemed to be more active for the reaction than the single protein extracted from the buffer.

It seems clear that both protein aggregation and water content are key parameters in determining the enzyme activity. In order to assess if the CMT-KDP HIP complexes formed in PFMC are catalytically active, a CMT catalysed transesterification reaction was studied.

Transesterification of *N*-acetyl-L-phenylalanine ethyl ester

The activity of α-chymotrypsin (CMT) solubilised in either hexane (CMT-AOT) or hexane-PFMC (CMT-KDP) for the transesterification of *N*-acetyl-L-phenylalanine ethyl ester (APEE) with *n*-butanol or *rac*-2-butanol was investigated. The activity of the solubilised protein was compared to that

of the suspended protease (native CMT) and control reactions with only the surfactants: AOT in hexane; or KDP in PFMC were also set up. The results are summarized in Fig. 4.

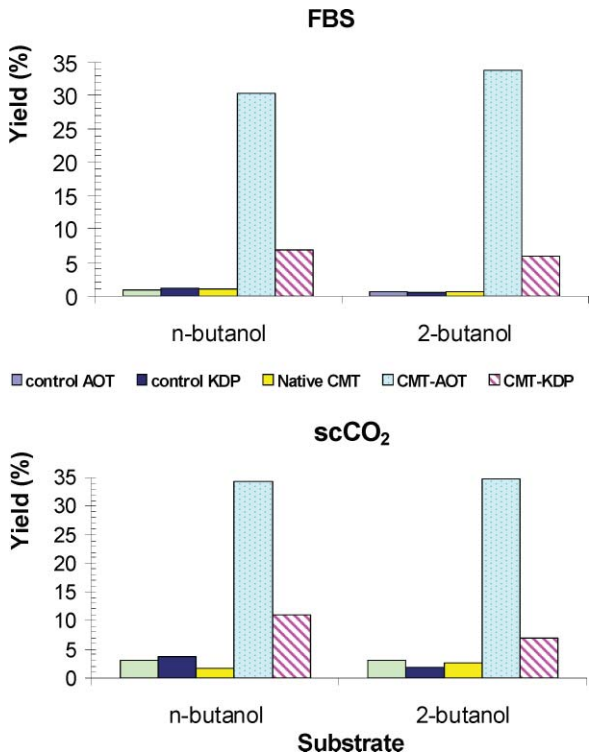


Fig. 4 Transesterification of APEE (4.2 mM) with either *n*-butanol or *rac*-2-butanol (0.42 mM) catalysed by surfactant AOT only (□) or KDP only (□) or native CMT (□) or solubilised CMT-AOT (□) or CMT-KDP (□) (0.01 mM) at 40 °C with stirring for two hours in the FBS hexane-PFMC v/v 1 : 1 in a 2 mL septum sealed glass vessel or in $scCO_2$ (100 bar) in a 8.5 mL high pressure stainless steel batch reactor. Yields were estimated by GLC equipped with a RTX-5 Fast column. Note that although CMT-KDP is not as active as CMT-AOT, it has higher activity than the native enzyme. However, unlike CMT-AOT, CMT-KDP can be recycled.

The HIP solubilised CMT exhibited higher activity than the suspended enzyme for the transesterification of APEE with the two alcohols tested in both a FBS and $scCO_2$. Under homogeneous conditions (hexane–PFMC and $scCO_2$), the dispersion of the enzyme–surfactant complexes into the reaction medium as well as the contact between the enzyme and the substrates are believed to be increased, thus enhancing the yield of the reaction.[13] With both alkyl alcohols tested, the yields of the ester products are slightly higher in $scCO_2$ compared to the hexane–PFMC which might be due to better mass transfer of substrates to the enzyme active site in $scCO_2$.

The CMT-AOT complexes in hexane appear to be around three times more active than the CMT-KDP complexes in PFMC and this trend was observed in both $scCO_2$ and hexane–PFMC. It is believed that the protein adopts an active configuration[14,16,17] in both media tested and that the difference in activity observed between CMT-AOT and CMT-KDP could be due to a difference in the accessibility of the active sites in the enzyme complexes formed with AOT and with the larger surfactant KDP restricting access more.

As explained above (see Fig. 3), particles of CMT-KDP in PFMC were found to form aggregates of a diameter of 25 nm, whereas the estimated size for CMT-AOT complexes (6.0 nm) suggested the presence of individual CMT molecules surrounded by the surfactant AOT in hexane. This difference in the particle size might explain the higher activity observed for CMT solubilised in hexane when compared to that in PFMC, as the number of accessible active sites in the latter will be much reduced as it is aggregated.

In the paper published by Akbar *et al.*, however, subtilisin Carlsberg or CALB enzyme aggregates formed were found to be more active than individual molecules extracted from the buffer for the transesterification of bergenin with vinyl butyrate in different organic solvents.[27] Their study and our results both demonstrate that solubilised enzymes aggregates can be more active than the suspended protein. Possibly, aggregation may provide a physical protection of the enzymes, thus maintaining them in an active form.[27]

The water content of enzyme–surfactant preparations is also a key factor in the activity of enzymes in non-aqueous media. This was determined using coulometric Karl Fisher titrations (see Table 2) and less than 1 ppm of water was detected for CMT-KDP in PFMC compared to about 50 ppm for CMT-AOT in hexane. The higher water content of CMT-AOT particles might also lead to the higher catalytic activity observed compared to CMT-KDP. Interestingly, our results suggest that an amount of water less than 1 ppm is sufficient to permit the formation of catalytically active protein-surfactant (CMT-KDP) particles.

The reusability of CMT-KDP dissolved in PFMC was investigated for the transesterification reaction in the fluorous biphasic system (hexane–PFMC) and in $scCO_2$ (100 bar) for 2 hours at 40 °C. The results achieved are presented in Fig. 5. The solubilised protease CMT-KDP in PFMC was successfully reused for the transesterification of APEE with both alcohols tested, and no loss of activity was observed over four cycles in the hexane–PFMC biphasic system or in $scCO_2$. CMT-KDP in PFMC exhibited good stability in both media investigated; in particular, its activity in $scCO_2$ did not appear to be affected by the pressurisation and depressurisation procedures.[13]

Conclusions

The technique of hydrophobic ion pairing (HIP) has been successfully used to solubilise α-chymotrypsin (CMT) using fluorinated surfactant KDP in PFMC. The transesterification of APEE catalysed by the HIP solubilised protease has been investigated in FBS and $scCO_2$. Under comparable conditions, we have shown that CMT solubilised in either hexane or PFMC *via* HIP gives higher product yields than the suspended enzyme. The CMT-KDP surfactant enzyme complexes which form catalytically active CMT-KDP aggregates in PFMC retain their activity over at least four reaction cycles, suggesting long-term recyclability.

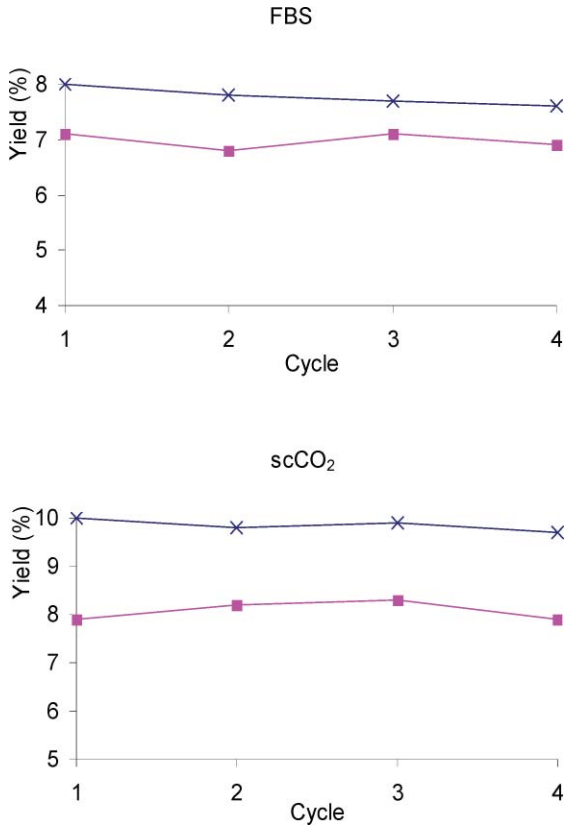


Fig. 5 Demonstration of the efficient recycling of CMT-KDP in PFMC for the transesterification of APEE with *n*-butanol (×) or *rac*-2-butanol (■) in the FBS hexane–PFMC and $scCO_2$ (100 bar) at 40 °C for two hours. Yields of were estimated by GLC equipped with a RTX5-Fast column.

Acknowledgements

We wish to thank Dr Helen R. Hobbs for the preliminary work performed on this project. We are also grateful to all our colleagues in the Clean Technology Group at the University of Nottingham, the School of Chemistry Workshop, Mark Guyler and Paul Hamley for their help and technical support. Finally, we thank Marie Curie Programme SubClean Probiomat (MEST-CT-2004-007767) for funding.

References

1 H. R. Hobbs and N. R. Thomas, *Chem. Rev.*, 2007, **107**, 2786–2820.
2 L. A. S. Gorman and J. S. Dordick, *Biotechnol. Bioeng.*, 1992, **39**, 392–397.
3 Y. Okahata, A. Hatano and K. Ijiro, *Tetrahedron: Asymmetry*, 1995, **6**, 1311–1322.
4 A. Gupte, R. Nagarajan and A. Kilara, *Ind. Eng. Chem. Res.*, 1995, **34**, 2910–2922.
5 D.-V. Unen, J. F. J. Engbersen and D. N. Reinhoudt, *Biotechnol. Bioeng.*, 2001, **75**, 154–158.
6 R. Bovara, G. Carrea, A. M. Gioacchini, S. Riva and F. Secundo, *Biotechnol. Bioeng.*, 1997, **54**, 50–57.
7 J. J. Lalonde, C. Govardhan, N. Khalaf, A. G. Martinez, K. Visuri and A. L. Margolin, *J. Am. Chem. Soc.*, 1995, **117**, 6845–6852.
8 H. R. Hobbs, B. Kondor, P. Stephenson, R. A. Sheldon, N. R. Thomas and M. Poliakoff, *Green Chem.*, 2006, **8**, 816–821.
9 P. Beier and D. O'Hagan, *Chem. Commun.*, 2002, 1680–1681.
10 H. R. Hobbs, *PhD Thesis*, 2006, University of Nottingham, UK.
11 D.-J. Unen, F. J. Johan and D. N. Reinhoudt, *Biotechnol. Bioeng.*, 2001, **75**, 154–158.
12 V. M. Paradkar and J. S. Dordick, *J. Am. Chem. Soc.*, 1994, **116**, 5009–5010.
13 H. R. Hobbs, H. M. Kirke, M. Poliakoff and N. R. Thomas, *Angew. Chem., Int. Ed.*, 2007, **46**, 7860–7863.
14 J. D. Meyer, B. S. Kendrick, J. E. Matsuura, J. A. Ruth, P. N. Bryan and M. C. Manning, *Int. J. Pept. Protein Res.*, 1996, **47**, 177–181.
15 J. D. Meyer, J. E. Matsuura, B. S. Kendrick, E. S. Evans, G. J. Evans and M. C. Manning, *Biopolymers*, 1995, **35**, 451–456.
16 V. M. Paradkar and J. S. Dordick, *Biotechnol. Bioeng.*, 1994, **43**, 529–540.
17 J. D. Meyer and M. C. Manning, *Pharm. Res.*, 1998, **15**, 188–193.
18 M. E. Powers, J. Matsuura, J. Brassell, M. C. Manning and E. Shefter, *Biopolymers*, 1993, **33**, 927–932.
19 S. S. Adkins, H. R. Hobbs, K. Benaissi, K. P. Johnston, M. Poliakoff and N. R. Thomas, *J. Phys. Chem. B*, 2008, **112**, 4760–4769.
20 The extinction coefficient of the protein was calculated using the ProtParm utility (http://www.expasy.org/tools/protparam.html); C. N. Pace, F. Vajdos, L. Fee, G. Grimsley and T. Gray, *Protein Sci.*, 1995, **4**, 2411–2423.
21 The X-ray structure is available from the world-wide protein data bank, (www.wwpdb.org): H. M. Berman, K. Henrick and H. Nakamura, *Nat. Struct. Biol.*, 2003, **10**, 980.
22 A. Dominguez, A. Fernandez, N. Gonzalez, E. Iglesias and L. Montenegro, *J. Chem. Educ.*, 1997, **74**, 1227–1231.
23 D. R. Lide, *CRC Handbook of Chemistry and Physics: A Ready Refererence Book of Chemical and Physical Data*, 85th edn, CRC Press, Boca Raton, Florida, USA, 2004.
24 http://www.allaboutinstruments.com/pdf/mitsubishi_karl_fisher_moisture.pdf, accessed January 2007 .
25 S. M. Howdle, P. Grebenik, R. N. Perutz and M. P., *J. Chem. Soc., Chem. Commun.*, **1989**, 1517–1519.
26 Y. L. Khmelnitsky, S. H. Welch, D. S. Clark and J. S. Dordick, *J. Am. Chem. Soc.*, 1994, **116**, 2647–2648.
27 U. Akbar, C. D. Aschenbrenner, M. R. Harper, H. R. Johnson, J. S. Dordick and D. S. Clark, *Biotechnol. Bioeng.*, 2007, **96**, 1030–1039.