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Stability of protein-coated microcrystals in organic solvents

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Abstract

Previously we reported a new high activity biocatalyst for use in organic media, termed *protein-coated microcrystals* (PCMC) [M. Kreiner, B.D. Moore, M.C. Parker, Chem. Commun. 12 (2001) 1906]. These novel biocomposites consist of water-soluble micron-sized crystalline particles coated with the given biocatalyst(s). Here we have looked at the stability of PCMC and their catalytic behaviour as a function of temperature in different organic media.

PCMC show very good long-term stability at room temperature, when stored as suspensions in 1-propanol/1 wt.% H_2O. *Candida antarctica* lipase B and subtilisin *Carlsberg* (SC) in PCMC form retained nearly 90% of their initial activity after 1 year at room temperature (RT). The effects of temperature on the catalytic activity of SC-PCMC are solvent-dependant. In 1-propanol/1 wt.% H_2O, the initial rate increased when the temperature was elevated from 25 to 60 °C, whereas in acetonitrile/1 wt.% H_2O, SC-PCMC lost activity. The operational stability of PCMC is also solvent-dependant. In 1-propanol/1 wt.% H_2O, SC-PCMC lost only 16% of the initial activity after five batch cycles. Rather poor stability was found for SC-PCMC in THF/1% (v/v) H_2O and acetonitrile/1% (v/v) H_2O, with a rapid loss of activity within 4 h in a continuous flow reactor. However, during the next 4 days only a slow further deactivation was observed.

Keywords: Protein-coated microcrystals (PCMC); Stability; Organic solvent; Subtilisin; Lipase

1. Introduction

The tremendous potential of enzymes as practical catalysts is well recognised [2–4]. In particular, their ability to act efficiently and highly selectively under mild conditions, such as ambient temperatures, near neutral pH and atmospheric pressures makes them ideal catalysts. Switching from the natural aqueous environment to employing enzymes in non-aqueous media offers, particularly from a synthetic perspective, many advantages [5]. Some of these include dramatically higher substrate solubility; the ability to use enzymes synthetically rather than degradatively and the capability to modify native selectivity by simply tailoring the reaction medium rather than the enzyme itself.

However, enzymes can frequently show lower reaction rates in organic media when compared to their respective activities in aqueous solution [6,7]. Over the last decade, several techniques have been developed to ameliorate this loss of catalytic activity, including immobilisation [8–10]; co-lyophilisation with lyoprotectants [11] and salts [12]; imprinting with substrates and substrate analogues [13]; additives [14] and other enzyme pre-treatments that positively affect the enzyme's history [15–17] as well as cross-linked enzyme crystals [18] and aggregates [19]. Previously, we reported new high activity biocatalysts for use in organic media, both polar and nonpolar solvents [1]. We termed this novel system: protein-coated microcrystals (PCMC). PCMC consist of water-soluble micron-sized particles, which are coated with the biocatalyst(s). PCMC are formed in a rapid 1-step process that simultaneously dehydrates and immobilises the protein on the micro-crystal surface, and Fig. 1 provides a simple illustration of the process. As can be seen from Fig. 1, the process includes a rapid dehydration step, whereby >90% of the water is removed from the immediate enzyme environment in seconds [20] and therefore, can obviate the need for

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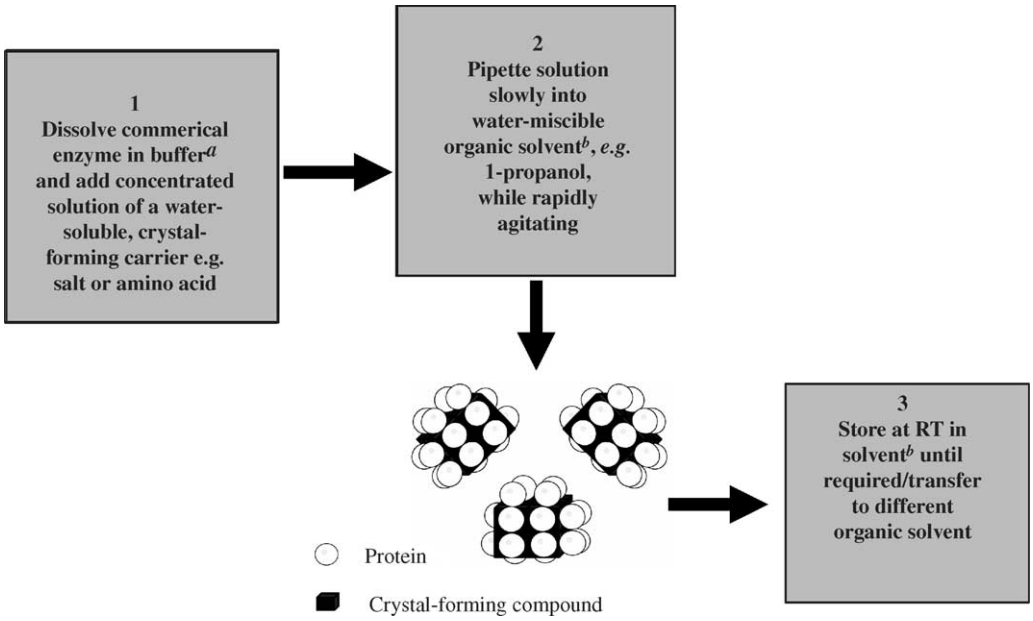

Fig. 1. Process scheme for preparation of protein-coated microcrystals: [a]at pH optimum; [b]preferably containing 1 wt.% H_2O.

lengthy drying processes, such as freeze-drying. The fine suspensions obtained have very useful properties, such as: ease of handling and accurate dispensing as a suspension and we have previously demonstrated considerably enhanced activity of proteases and lipases using this process [1] when compared to conventional freeze-drying methodology and the *as received* enzyme powders. For use in organic media (and importantly this includes a wide range of polar solvents), the precipitating solvent, if not appropriate for the reaction conditions, e.g. low substrate solubility, can be decanted and replaced by another reaction solvent. Alternatively, for aqueous applications, the PCMC can be rapidly re-dissolved back into aqueous solution. This suggests that PCMC may find widespread application as a generic method for the production of biocatalysts with predictable morphology, high activity and excellent handling ability in biocatalyst screening programs, amongst others.

To find widespread use, clearly good retention of activity of a biocatalyst preparation is of prime importance. Therefore our focus in this work was to investigate the stability of PCMC with three main aspects: firstly, room temperature stability with regard to their shelf-life; secondly, the catalytic activity as a function of temperature and finally their operational stability.

2. Experimental

2.1. Materials

Subtilisin *Carlsberg* (SC) protease, Type VIII from *Bacillus licheniformis*, 11.7 units mg^{-1} solid (P5380) was obtained from Sigma (Poole, UK). *Candida antarctica* lipase L-2 (CALB), *Mucor miehei* lipase L-9 (MML) and *Pseudomonas* sp. lipase L-6 (PSL) were obtained from the *Lipases and Esterases Screening Set 2* (product no. 1859366) from Roche Diagnostics (Germany). The cross-linked subtilisin crystals ChiroCLEC^{TM}-BL (lot: 98016) were purchased from Altus Biologics Inc. (USA). Propanol rinsed enzyme preparations (PREPS) were prepared as reported by Fernandes and Halling [21]. *N*-Acetyl-L-tyrosine ethyl ester (A6751) was also purchased from Sigma. All solvents (HPLC grade) were from Sigma–Aldrich and were used without any further purification. The water content of the solvent was set by adding water and the final water content was determined using a Karl Fisher 684 KF Coulometer (Metrohm, Switzerland). Potassium sulphate (99%) was from Sigma–Aldrich (UK).

2.2. Preparation of PCMC

The procedure used routinely for producing PCMC was as follows (see also Fig. 1): subtilisin *Carlsberg* (4 mg) was usually dissolved in 100 μl Tris–HCl (10 mM, pH 7.8) and then mixed with 300 μl of a saturated aqueous solution of K_2SO_4 (carrier material). This solution was then added drop-wise to a vial containing 6 ml of 1-propanol/1 wt.% H_2O under constant shaking. The resulting PCMC (rectangular crystals with dimensions of 0.1–5 μm, [1]) were separated from the solvent by centrifugation and immediately washed with 1-propanol/1 wt.% H_2O. Finally, PCMC were re-suspended in 1-propanol/1 wt.% H_2O until required. Lipase-PCMC were prepared using the same procedure as described above, however the enzyme was first dissolved in Na-phosphate buffer (50 mM, pH 7.0). The theoretical protein content of these PCMC, calculated as (mg of initial protein) per (mg of initial protein + carrier material), was 11.8 wt.%.

2.3. Activity assays

2.3.1. Assay for catalytic activity in organic solvent

The model reaction for subtilisin *Carlsberg* was the transesterification of *N*-acetyl-L-tyrosine ethyl ester (10 mM) with 1-propanol (1 M). The reaction solvent contained 1 wt.% H_2O. The model reaction for lipases was the kinetic resolution of 1-phenylethanol (0.1 M) using vinyl acetate (0.3 M) as acyl donor. Dry *tert*-butyl methyl ether was used as the reaction solvent for lipases, except for the solvent compatibility studies. Immediately before use in the assay the lipase-PCMC were washed with the reaction solvent used in order to remove 1-propanol. Reaction details and HPLC analysis for the two assays were as described previously [22]. The catalytic rates are based on mg of initial protein used for the preparation of the PCMC. In this study, all reactions were carried out at least in triplicate.

2.3.2. Determination of the catalytic activity of SC-PCMC in aqueous solution after recovery from a continuous flow reactor

At the end of a continuous flow reaction experiment, subtilisin PCMC were recovered from the reactor tube by rinsing with 1 ml of 1-propanol prepared at the same water activity (a_w) as in the reaction solvent. Then the resultant 1 ml suspension was homogenised by gentle stirring and samples of 100 μl were transferred into an eppendorf tube. 1-Propanol was removed after centrifugation, and 100 μl of Tris–HCl buffer was added to dissolve the microcrystals. These were immediately transferred to 5 ml of a freshly prepared assay solution pre-equilibrated at 25 °C. The dissolved crystals were then automatically titrated in the pH-stat apparatus to measure catalytic activity. The assay solution consisted of 10 mM *N*-acetyl-L-tyrosine ethyl ester (first dissolved in acetonitrile to give 5% of the total volume) in 20 mM Na-phosphate buffer at pH 7.8 [21]. Reproducible activity measurements (2–3) were obtained for each set of experiments. The catalytic activity measured was then compared with the activity of unused PCMC from the storage suspension in 1-propanol/1 wt.% H_2O, which was determined at the same aqueous assay conditions.

2.4. Stability studies

2.4.1. Long-term stability at RT

PCMC were prepared and stored at RT as a suspension in 1-propanol containing 1 wt.% H_2O. For experiments where enzyme precipitated in the absence of carrier was tested, the precipitation conditions for these preparations were the same as for PCMC, except that H_2O was used instead of the aqueous carrier solution. Increasing amounts of H_2O (up to 10.5 wt.%) were added to the storage solvent in order to study the effect of the water content of the storage solvent on the activity of SC-PCMC.

2.4.2. Study of temperature effects on activity

The transesterifications of *N*-acetyl-L-tyrosine ethyl ester (10 mM) with 1-propanol (1 M) using SC/K_2SO_4-PCMC as catalyst were conducted at 25, 40, 50 and 60 °C. The solvents used were 1-propanol and acetonitrile, both containing 1 wt.% H_2O.

2.4.3. Operational stability studies

The stability of the PCMC was tested either in a continuous process or by performing subsequent batch reaction cycles. The continuous flow reactions with PCMC, CLECs and PREPs were performed using a continuous micro-reactor at a flow rate of 3 ml h^{-1}. The temperature was set to 22 °C by using a temperature-controlled incubator. The solvent in the feed contained the substrates: *N*-acetyl-L-tyrosine ethyl ester (10 mM) and 1-propanol (1 M). The set-up of the reactor and reaction conditions were as described previously [21]. Acetonitrile or THF were used as a reaction solvent. For experiments with PCMC the water content of the solvents was 1% (v/v). (This corresponds to a water activity a_w of 0.22 or 0.40, respectively). For experiments with PREPs and CLECs, the a_w of both solvents was 0.22. (This corresponds to a water content of 1% (v/v) or 0.45% (v/v), respectively.) For batch reactions, SC/K_2SO_4-PCMC were subjected to repeated conversions of the substrate *N*-acetyl-L-tyrosine ethyl ester (10 mM) with 1-propanol (1 M, expect when 1-propanol was used as reaction solvent). The solvents used were either acetonitrile (AcN), tetrahydrofuran (THF), *tert*-butanol (*t*-BuOH) or 1-propanol, all set to a water content of 1 wt.%. Between the batch cycles the PCMC were washed with the reaction solvent to remove any substrate and product.

3. Results and discussion

3.1. Storage stability of PCMC at RT

Earlier stability tests of subtilisin *Carlsberg*-PCMC, suspended in 1-propanol/1% H_2O, revealed that this biocatalyst preparation has the potential to provide a very long shelf-life for enzymes at RT [1]. The aim of this study was to explore, if these findings could be extended to other enzymes. In addition, we wanted to investigate how critical the water content of the storage solvent or the immobilisation of the enzyme onto the carrier crystals are for long-term storage. The long-term stability of PCMC at RT, when stored as a suspension in 1-propanol containing 1 wt.% H_2O, was tested with two lipases and SC. K_2SO_4 was used as carrier for PCMC, because it adsorbs very little water, is cheap and readily available, and further we have found it produces fine particles that can be handled very easily [1].

The catalytic activities in organic solvent of PCMC were measured at defined time intervals and this data is shown in Fig. 2. Both lipases, *Candida antarctica* lipase B (CALB) and *Pseudomonas* sp. lipase (PSL), showed good stability. CALB-PCMC retained 88% of the initial activity within 12

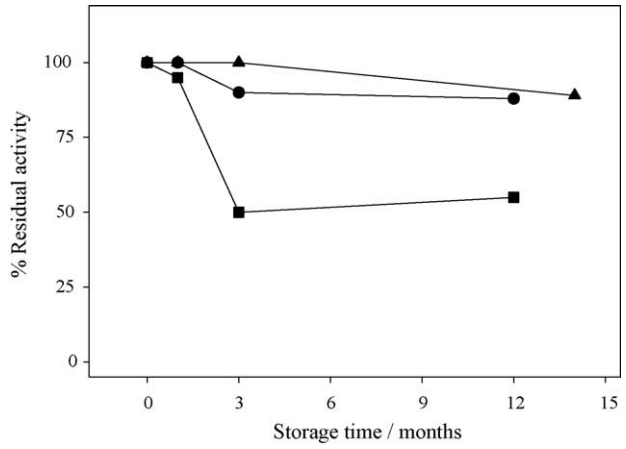

Fig. 2. Long-term stability of PCMC at RT when stored in 1-propanol/1 wt.% H_2O. PCMC were made using K_2SO_4 as support and 1-propanol/1 wt.% H_2O as precipitating solvent. Shown are the retained catalytic activities (percentage of initial activity based on percentage conversion) in organic solvent. Reaction conditions were as described in Section 2. (▲) PCMC-subtilisin *Carlsberg*; (●) PCMC-CALB; (■) PCMC-*Pseudomonas* sp. lipase.

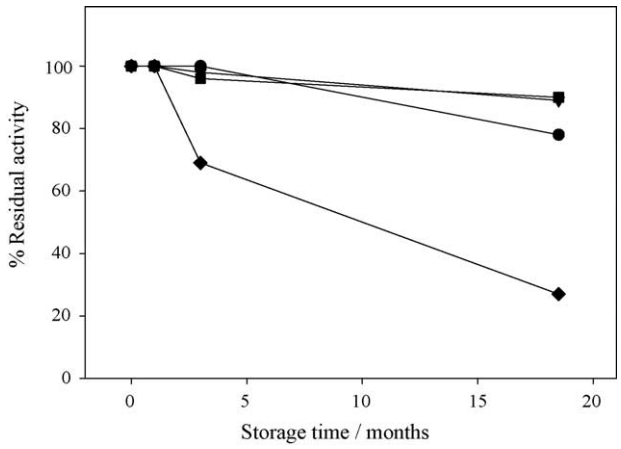

Fig. 3. Long-term stability of subtilisin *Carlsberg*/K_2SO_4-PCMC at RT. Effect of water content of the storage solvent 1-propanol. Shown are the catalytic activities (percentage of initial activity based on percentage conversion after 30 min) in acetonitrile/1 wt.% H_2O. Reaction conditions were as described in Section 2. (●) 1.0 wt.% H_2O; (▼) 2.6 wt.% H_2O; (■) 5.0 wt.% H_2O; (♦) 10.5 wt.% H_2O.

months. *Pseudomonas* sp. lipase was found to be generally less stable than CALB, losing 50% of the activity within 1 year when stored as PCMC. With all lipases, the largest drop in activity was found within the first 3 months, thereafter the activity was constant or decreased only slowly, regardless of the type of enzyme formulation. Subtilisin *Carlsberg* PCMC also showed very good stability at RT, retaining 89% of the activity after 14 months.

In order to observe, if the good shelf-life of enzymes presented in the PCMC form were as a result of the immobilisation onto the K_2SO_4 carrier, the long-term stability of SC, CALB and PSL, precipitated in 1-propanol in the absence of carrier, was also investigated. The enzymes were treated in the same way as for preparation of PCMC, except in this instance no carrier was added for the precipitation. Interestingly, these precipitated enzyme preparations were as active and as stable in 1-propanol/1 wt.% H_2O as when stored in PCMC form. This suggests that the immobilisation on the carrier is not a critical parameter for improved long-term stability. On the other hand, protein-coated microcrystals offer the advantage of improved handling properties, such as accurate dispensing of the biocatalyst suspension, and easier downstream processing, compared to proteins precipitated in 1-propanol in the absence of carrier crystals. These results indicate that the storage of enzymes in 1-propanol/1 wt.% H_2O may be, more generally, a valuable method to store proteins at RT by restricting the proteins mobility/flexibility and thus preventing e.g. autolysis.

The availability of H_2O is generally critical for proteins/enzymes in organic solvent [5,23]. There is clearly a trade-off here: whereas a small amount of water may be necessary to keep the enzyme flexible, higher water concentrations in the system can promote enzyme degradation/denaturation. For example, subtilisin *Carlsberg* and α-chymotrypsin are known to be prone to autolysis [24], and therefore the presence of high levels of H_2O will favour this process. Therefore we tested the shelf life of SC/K_2SO_4-PCMC in 1-propanol containing different amounts of water (1.0–10.5 wt.%). As shown in Fig. 3, water contents up to 2.6% had no effect on the long-term stability, even at a water content of 5% only a 10% drop in activity was observed. However, the presence of 10.6 wt.% H_2O ($a_w = 0.76$) in the storage solvent 1-propanol resulted in a significant loss of activity with increasing storage time. Approximately 30% of the activity was lost within 3 months, and within 18 months this had increased to 73%.

SC-PCMC and lipase-PCMC show very good long-term stability at RT, when suspended in 1-propanol/1 wt.% H_2O. This is unexpected, because enzymes typically require storage at reduced temperature. Prolonged shelf-life at RT provides a much higher level of convenience. If enzymes could be used in the same 'off-the-shelf' manner as chemical catalysts, i.e. they could be stored without the need for refrigeration, this would clearly be advantageous and would ultimately attract use from a wider community. Employing lipases and proteases as PCMC is an appropriate method for achieving this as such biocatalyst preparations show good storage stability in 1-propanol (containing small amounts of H_2O; 1–2.5 wt.%), enhanced activity in organic solvent, and are easy to dispense. These properties may be extendable to PCMC prepared with other enzymes.

3.2. Solvent compatibility

The type of solvent used can be critical for a biocatalytic reaction, because the solvent affects both the enzyme's stability as well as its specificity. It is generally believed that enzymes are relatively stable in nonpolar solvents since they are kinetically trapped. More hydrophilic solvents are usu-

Table 1
Catalytic activity of SC/K_2SO_4-PCMC in different organic solvents (all containing 1.0 wt.% H_2O)

Reaction solvent	Rate (nmol min^{-1} mg^{-1})	% conversion after 2 h	% conversion after 21 h
THF	68 ± 3	3	–
Acetonitrile	136 ± 16	16	41
tert-Butanol	93 ± 6	23	94
1-Propanol	121 ± 22	35	100

Transesterification of *N*-acetyl-L-tyrosine ethyl ester with 1-propanol.

ally inferior, because they have a stronger tendency to strip tightly bound water from the enzyme molecules if used at a fixed water content. Therefore the catalytic behaviour of a new biocatalyst preparation in more polar solvents is of particular interest. Here, the performance of PCMC prepared with SC and K_2SO_4 as carrier was tested in the following commonly used solvents: 1-propanol, *tert*-butanol, acetonitrile and THF. The reaction studied was the transesterification of *N*-acetyl-L-tyrosine ethyl ester with 1-propanol. All the solvents contained 1 wt.% H_2O. As shown in Table 1, both alcohols, 1-propanol and *tert*-butanol, together with acetonitrile allowed a fast conversion of the substrate. SC showed the highest initial activity in acetonitrile/1 wt.% H_2O, however, this solvent quickly led to inactivation of the enzyme with increasing contact time. Therefore the percentage conversion versus time profile shows a curvature—illustrating this point, whereas with the other solvents tested, linear profiles were obtained. In *tert*-butanol, 94% of the substrate was converted after 21 h, and 1-propanol allowed complete conversion of the substrate within that time. Unexpectedly, SC showed low activity in THF. This solvent allowed only very slow conversion: only 3% of the substrate were converted within 2 h.

Converting the water content of 1 wt.% for each reaction solvent, as used here, into the corresponding water activities a_w (1-propanol/1% H_2O: $a_w = 0.15$, AcN/1% H_2O: $a_w = 0.22$, *t*-BuOH/1% H_2O: $a_w = 0.27$, THF/1% H_2O: $a_w = 0.43$) [25] shows that there is no correlation between catalytic activity and a_w. This suggests that the differences in activity and particularly the inactivation of SC-PCMC in actonitrile/1% H_2O with time are not only as a result of the availability of water for the enzyme, but are caused by solvent specific effects.

The catalytic activity of PCMC prepared with lipases and K_2SO_4 was tested in acetonitrile/0.04 wt.% H_2O and dry *tert*-butyl methyl ether. The activities in *tert*-butyl methyl ether were generally higher than in acetonitrile. The activity demonstrated by CALB in acetonitrile was only 20% of that in *tert*-butyl methyl ether (based on percentage conversion after 1 h). PCMC of *Mucor miehei* lipase showed a conversion of 14% in *tert*-butyl methyl ether after 1 h [1], whereas in acetonitrile less than 1% conversion of the substrate was observed after 46 h. Compared to *tert*-butyl methyl ether, the activity of *Pseudomonas* sp. lipase in PCMC form decreased from 52% conversion (after 30 min) to 9% in acetonitrile.

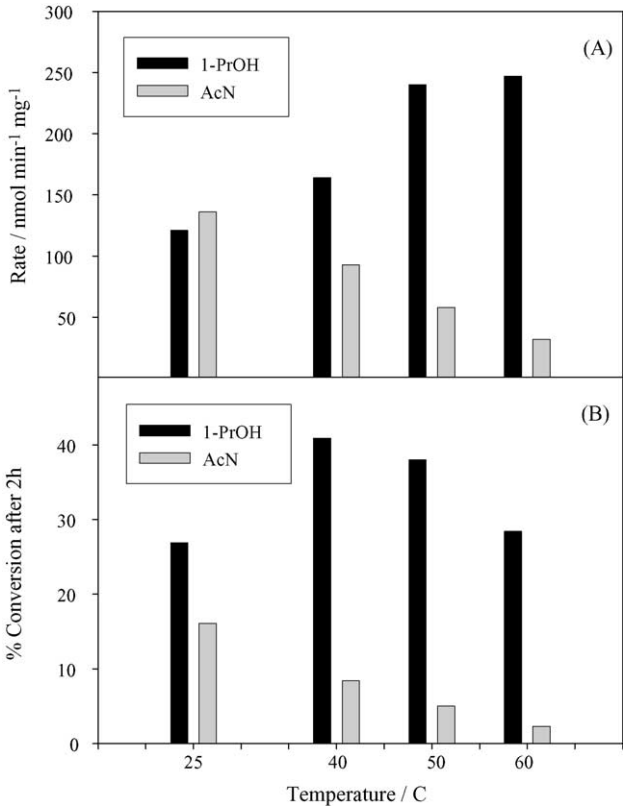

Fig. 4. Effects of temperature on the catalytic activity of SC/K_2SO_4-PCMC in acetonitrile/1 wt.% H_2O (grey bars) and 1-propanol/1 wt.% H_2O (black bars). Shown are the initial rates (A) and the percentage conversion of *N*-acetyl-L-tyrosine ethyl ester after 2 h (B).

The lower activities observed in acetonitrile may be due to the increased water content.

3.3. Study of temperature effects on activity

The thermal stability of enzymes is an important feature for the application of the biocatalysts in a commercial setting. Therefore, the catalytic activity of SC/K_2SO_4-PCMC was tested at different temperatures (25–60 °C). Two reaction solvents were used: 1-propanol and acetonitrile, both containing 1 wt.% H_2O. As can be seen in Fig. 4A, the effects of temperature on SC/K_2SO_4-PCMC activity were different in the two solvents tested. In 1-propanol the activities obtained were as expected from simple kinetics: they increased with increasing temperature. The initial rate approximately doubled by increasing the temperature from 25 to 50 °C. Increasing the temperature further to 60 °C, only resulted in a slight further increase in catalytic activity. When examining the conversions after 2 h (Fig. 4B), it shows that even though the highest initial rate was obtained at 60 °C, this temperature leads to the inactivation of the enzyme with time. The maximum conversion after 2 h was observed at a reaction temperature of 40 °C. In contrast to 1-propanol/1 wt.% H_2O, the activity in acetonitrile/1 wt.% H_2O decreased with increasing temperature. At 60 °C the initial rate was only 23% of

that measured at 25 °C. Similarly, the final conversion (after 2 h) decreased from 16% at 25 °C to 2% at 60 °C. The inactivating effect of acetonitrile as such was clearly enhanced with increasing temperature. This may be explained by the fact that SC is inactivated fairly quickly in acetonitrile even at RT, leading to a strong curvature of the reaction profile.

Freeze-dried SC showed very low activity at all the temperatures tested (<1% conversion in acetonitrile/1 wt.% H_2O after 3 h). Subtilisin in aqueous solution (pH 8.5) is quite stable up to 50 °C, but then loses activity rapidly with increasing temperature [26]. At 60 °C approximately 90% of the activity is retained after 60 min, at 65 °C this is 20% and at 70 °C the enzyme is completely inactivated after only 35 min. Compared to the reaction temperature of 25 °C SC-PCMC showed no loss of activity in 1-propanol/1 wt.% H_2O at 60 °C. On the contrary, they were more active than at 25 °C (about 210% of the activity). Clearly, it is well known that the temperature stability of enzymes is often increased in low water conditions [27,28], thought to be as a consequence of increased rigidity of the enzyme due to a lack of H_2O.

To summarize, SC/K_2SO_4-PCMC showed different responses to elevated reaction temperatures depending on the reaction solvent used. Whereas the inactivating effect of acetonitrile at RT was increased at higher temperatures, SC-PCMC show good temperature stability, if the reaction solvent used does not already show inactivating effects on the enzyme at RT: with 1-propanol/1% H_2O, the initial rates increased with increasing temperature.

3.4. Operational stability

Perhaps the most important and realistic assessment of a biocatalyst's applicability for biotransformation, particularly from an economic viewpoint, is its operational stability. We were therefore interested to study the operational stability of PCMC in continuous flow operation and hence their industrial applicability and compare it to the stability of other high activity biocatalyst preparations, subtilisin 'propanol rinsed enzyme preparations' (PREPs) and 'cross-linked enzyme crystals' (CLECs). In CLECs, enzyme molecules are crystallised and cross-linked through covalent bonds, whereas in PREPs enzyme remains adsorbed on a silica surface interacting with silanol groups through non-covalent interactions. For comparison, in PCMC the enzyme molecules are situated on the surface of the carrier crystal, in this case salt crystals.

Fig. 5 shows the typical deactivation profiles of subtilisin-PCMC, -CLECs and -PREPs in AcN and THF. The deactivation profiles of the three subtilisin preparations showed two distinct phases of inactivation: a phase of rapid inactivation over the first 3–4 h of continuous flow, followed by a phase where the inactivation proceeded more slowly. The deactivation profiles of the three SC preparations were well represented by a sum of two exponential functions, $a(t) = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t)$ [21]. The deactivation parameters of the fittings are shown in Table 2. In a similar fashion to PREPs and CLECs, PCMC also lost more than 50% of

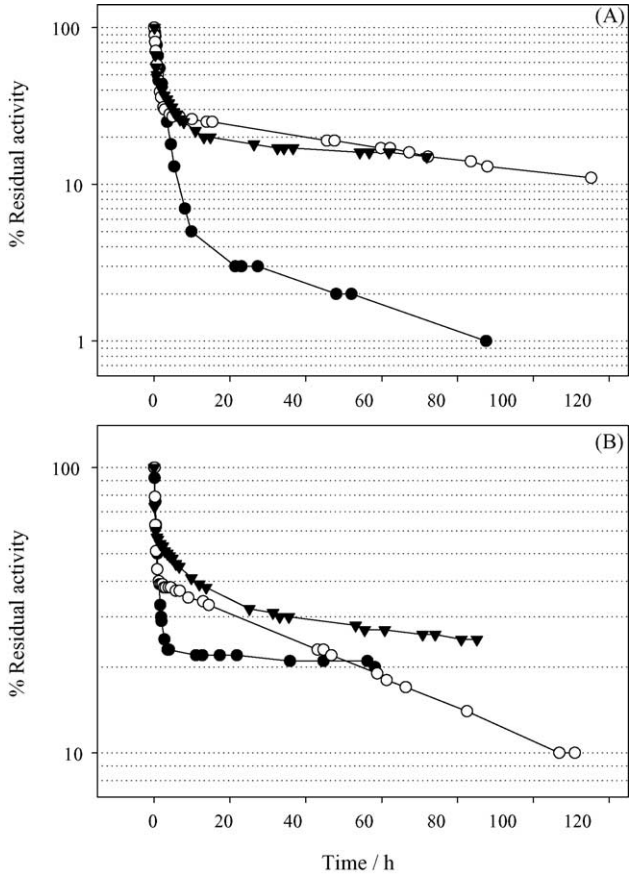

Fig. 5. Typical deactivation profiles of subtilisin/K_2SO_4-PCMC (▼) in a continuous flow reactor. Solvents used were (A) acetonitrile at $a_w = 0.22$ and (B) THF at $a_w = 0.40$. Comparison with deactivation profiles of subtilisin CLECs (◯), and PREPs (●) in (A) acetonitrile and in (B) THF, both at $a_w = 0.22$. Shown are fitted data.

the activity at the start of the continuous flow reaction in both solvents. In THF, the fraction of inactivated enzyme over the rapid phase is generally lower than in AcN (lower f value). But SC-PCMC showed similar rates of inactivation over the slower phase of inactivation (k_2) in both solvents. Amongst all the different preparations, PCMC showed the best operational stability over the second (slower) phase of inactivation with the lowest values of k_2 in AcN. However, SC-PCMC showed a similar fraction of inactivation (f) as SC-CLECs in both solvents. In contrast PREPs in AcN showed the highest fraction of inactivated enzyme over the rapid phase ($f = 0.92$). In THF, the fraction of inactivated enzyme was lower ($f = 0.76$) but still higher than both with CLECs ($f = 0.60$) and PCMC ($f = 0.68$). SC-CLECs, despite being highly active at the beginning of the continuous reaction, showed a pronounced loss of activity in THF even in the slower phase of inactivation (high k_2). Overall, PCMC showed the lowest rates of inactivation over the second, slower phase of inactivation. Keeping in mind that, despite a loss in activity during continuous reaction, PCMC are still two orders of magnitude more active after a reaction time of 4 days than lyophilised SC, and thus therefore makes them potentially, very useful biocatalysts for

Table 2
Operational stability of different subtilisin *Carlsberg* preparations in continuous flow experiments

Enzyme preparation	Solvent	Water activity (water content (%, v/v))	a_2[a] (nmol min^{-1} mg^{-1})	k_2[a] (10^{-3} h^{-1})	f[b]
PCMC	AcN	0.22 (1.0)	63 ± 2	3.5 ± 0.6	0.74 ± 0.01
CLECs		0.22 (1.0)	246 ± 34	8.4 ± 2	0.73 ± 0.04
PREPs		0.22 (1.0)	29 ± 9	17.3 ± 6.8	0.92 ± 0.02
PCMC	THF	0.40 (1.0)	15 ± 1	3.4 ± 0.5	0.68 ± 0.02
CLECs		0.22 (0.45)	39 ± 3	12.7 ± 1.7	0.60 ± 0.03
PREPs		0.22 (0.45)	54 ± 7	2.4 ± 1.8	0.76 ± 0.02

Fitted parameters from two-exponential model for deactivation of PCMC, CLECs and PREPs. Transesterification of *N*-acetyl-L-tyrosine ethyl ester with 1-propanol in either acetonitrile or THF.

[a] Activity decay was described by a two-exponential equation, $a(t) = a_1 \exp(-k_1 t) + a_2 \exp(-k_2 t)$, where $a(t)$ is the observed activity, a_1 and a_2 are the apparent specific activities for the components deactivated faster and slower respectively; and k_1 and k_2 are the corresponding first order rate constants. The faster exponential decay is represented only by relatively few initial points, and we found that k_1 was not reliably estimated. Hence a_2 and k_2 were determined by linear regression to the longer time points, before fitting the first exponential. Each of at least 2 replicate inactivation curves was fitted separately, and the average parameter value is quoted. Errors were estimated based on the fitting errors from each replicate, and the difference between replicates.

[b] Fraction of enzyme population deactivated in the first phase. f was calculated as $1 - a_2/a_0$, where a_0 was the initial activity determined experimentally.

biosynthetic applications. Furthermore, the fact that they are comparably stable after an initial loss of activity is a distinct advantage.

In order to observe, if the catalytic activity could be regained when returned to the initial conditions, PCMC recovered from reactors after a reaction time of four days were transferred to 1-propanol. Then their activity was measured in an aqueous assay. It was found that PCMC, that were inactivated during the continuous flow in AcN/1% (v/v) H_2O, did not regain activity when transferred to the aqueous assay solution. Hence, we concluded that the subtilisin PCMC inactivation was irreversible. The same irreversible inactivation was observed in subtlisin PREPs and CLECs recovered from the continuous reactors [21].

The stability of the catalyst using alcohols as reaction solvents was tested by performing subsequent reaction cycles of the transesterification of *N*-acetyl-L-tyrosine ethyl ester with 1-propanol in either 1-propanol/1 wt.% H_2O or *tert*-butanol/1 wt.% H_2O. For comparison with the continuous process, acetonitrile and THF as reaction solvents were included as well. Subjecting the enzyme to repeated batch reactions, including all workup and catalyst recovery operations between the reactions, and then monitoring any decrease in reaction rate for subsequent reaction allows assessment of all possible catalyst deactivation events including possible enzyme denaturation during recovery operations. The results of the batch recycling are shown in Fig. 6. In *tert*-butanol/1 wt.% H_2O, SC in PCMC form lost 27% of the activity after 1 cycle of 1 h. In the following cycles, the activity loss was small (<4%). Highest stability of SC-PCMC was found when 1-propanol/1 wt.% H_2O was used as reaction solvent. With this solvent there was no discernable loss of activity during the first four cycles of 1 h, in the following 2 cycles 16% of the activity was lost after each cycle. In acetonitrile/1 wt.% H_2O, approximately 60% of the activity is lost after each cycle (reaction time = 3 h). In THF/1 wt.% H_2O, SC-PCMC have lower activity than in acetonitrile/1 wt.% H_2O (see also Table 1), however they are more stable, with 70–80% of the initial activity retained after one cycle (reaction time = 3 h).

In summary, all subtilisin preparations studied showed rather poor operational stability during the continuous flow operation in aprotic organic solvents (THF and acetonitrile) with a more than 50% decrease in catalytic activity within the first 4 h. However, over the slower phase of inactivation (up to 4 days) subtilisin-PCMC showed the best stability when compared with SC-CLECs and SC-PREPs. This presents a clear advantage for the PCMC preparation. The SC-PCMC inactivation is irreversible, as they do not recover their catalytic activity when assayed in aqueous solution. Therefore deactivation is not solely due to dehydration. Inactivation of SC-PCMC can be significantly reduced by using protic solvents as reaction solvents (1-propanol, *tert*-butanol) as shown in batch recycling experiments.

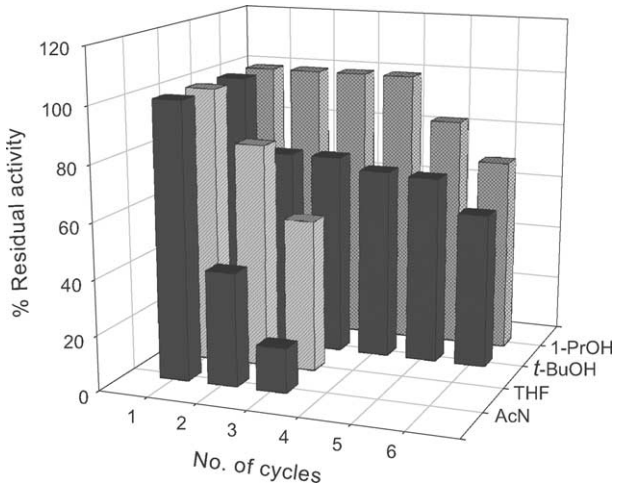

Fig. 6. Performance of SC/K_2SO_4-PCMC in batch recycle experiments. PCMC were subjected to repeated conversions of the *N*-acetyl-L-tyrosine ethyl ester. Between the reactions the PCMC were washed with the respective solvent. The reaction time in the alcohols/1 wt.% H_2O was 1 h and in acetonitrile/1 wt.% H_2O and THF/1 wt.% H_2O 3 h. Percentage residual activity is based either on percentage conversion after 30 min (3 h) when acetonitrile/1 wt.% H_2O (THF/1 wt.% H_2O) was used or on the initial rate for reactions in 1-propanol/1 wt.% H_2O or *tert*-butanol/1 wt.% H_2O.

4. Conclusions

1. PCMC prepared with subtilisin *Carlsberg* and several lipases show high long-term storage stability at RT, when stored as a suspension in 1-propanol/1 wt.% H_2O: 50–90% of the initial activity, depending on the enzyme, was retained after 1 year storage at RT. Immobilisation of the enzyme onto the carrier salt is not essential, but produces advantages for handling and further processing. Precipitating into or rinsing with 1-propanol/1 wt.% H_2O is not only a favourable way of drying enzymes, leaving them in a highly active form, but this solvent is also a very good storage agent for PCMC.
2. The effect of temperature on the activity of SC-PCMC depends on the reaction solvent. In 1-propanol, SC-PCMC show increased catalytic activity with increasing temperature (up to 60 °C). Equally, the inactivation of SC by acetonitrile is accelerated with increasing temperature, resulting overall in a reduced activity with increasing temperature in this solvent.
3. Although SC-PCMC show high activity in organic media together with high storage stability at RT, their operational stability during continuous flow operations in aprotic organic solvents is rather low, which would be a considerable drawback for many applications. However when compared with two other high activity biocatalyst preparations, PREPs and CLECs, subtilisin-PCMC showed the lowest rate of inactivation over the slower phase of inactivation in both acetonitrile and THF. Moreover, despite the initial loss of activity, PCMC are still two orders of magnitude more active after 4 days of continuous reaction in aprotic organic solvent than lyophilised SC. Alternatively, use of protic solvents, such as alcohols as reactions solvent allows better operational stability, as shown in batch recycle experiments.
4. Alcohols, particularly 1-propanol, are preferred reactions solvents for SC-PCMC, with regard to activity and stability: SC-PCMC show highest activity among the solvents tested as well as linear reaction profiles in this type of solvent, and the catalytic activity increases with increasing reaction temperature (up to 60 °C). Furthermore, suspending enzymes in 1-propanol/1% H_2O proved to warrant good long-term storage stability of enzymes at RT. This distinguishes 1-propanol/1% H_2O as a benign solvent for enzyme applications in organic solvent.

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