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Enzyme–Carbon Nanotube Conjugates in Room-temperature Ionic Liquids

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Abstract Room-temperature ionic liquids (RTILs) are intriguing solvents, which are recognized as "green" alternatives to volatile organics. Although RTILs are nonvolatile and can dissolve a wide range of charged, polar, and nonpolar organic and inorganic molecules, there remain substantial challenges in their use, not the least of which is the solvents' high viscosity that leads to potential mass transfer limitations. In the course of this work, we discovered that the simple adsorption of the bacterial protease, proteinase K, onto single-walled carbon nanotubes (SWNTs) results in intrinsically high catalytic turnover. The high surface area and the nanoscopic dimensions of SWNTs offered high enzyme loading and low mass transfer resistance. Furthermore, the enzyme–SWNT conjugates displayed enhanced thermal stability in RTILs over the native suspended enzyme counterpart and allowed facile reuse. These enzyme–SWNT conjugates may therefore provide a way to overcome key operational limitations of RTIL systems.

Keywords Room-temperature ionic liquids · Enzyme–SWNT conjugates · Diffusional limitations · Enzyme kinetics · Thermostability · Reusability

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Introduction

Recent advances in the use of enzymes in nonaqueous media have had a profound effect on biotransformations of interest to the chemical and pharmaceutical industries [1, 2]. In particular, the use of organic solvents as a reaction medium enables synthetic strategies heretofore impossible in aqueous media. Despite these advances, organic solvents suffer from several key drawbacks, including poor solubility of charged and highly polar compounds and high volatility. These limitations have prompted the design of specialized solvents, which offer the opportunity to remain nonaqueous in character yet overcome the drawbacks of an organic solvent. One such solvent class is the room-temperature ionic liquids (RTILs), which have emerged as useful solvents because of their negligible vapor pressure, high chemical and thermal stabilities, and ability to solubilize a wide range of charged, polar, and nonpolar species. The physical properties of RTILs, such as density, viscosity, melting point, polarity, and miscibility with water or organic solvents, can be altered by changing the anion or the substituents in the cation or both. These possibilities make RTILs at least a limited alternative to organic solvents [3, 4].

Room-temperature ionic liquids have been used as reaction media for a variety of enzymatic reactions. Examples include α-chymotrypsin-catalyzed aminoacid ester transesterification [5–7]; enantioselective lipase-catalyzed esterification, transesterification, perhydrolysis, and aminolysis reactions [8]; β-galactosidase-catalyzed galactosylation [9, 10]; thermolysin-catalyzed aspartame synthesis [11]; and peroxidase-catalyzed phenolic oxidation [12, 13]. This diverse repertoire of enzymatic reactions demonstrates the potential value of RTILs as novel media for enzymatic reactions. In addition, RTILs have been shown to support enhanced operational properties of enzymes, such as increased thermal stability [7, 14, 15].

Despite the preponderance of reports of enzymatic catalysis in RTILs, a common problem that plagues such systems is the low reactivity achieved. For this reason, we endeavored to develop more active formulations for use in RTILs. One particularly interesting formulation involves enzyme–carbon nanotube conjugates, which we have previously shown to result in more stable heterogeneous enzyme preparations under harsh conditions (e.g., in the presence of organic solvents and high temperatures) [16, 17]. This stabilization coupled with the very high surface area per unit mass of single-walled carbon nanotubes (SWNTs) without substantive intraparticle diffusional mass transfer limitations, as would be expected for similarly high surface area porous supports, make SWNTs uniquely suited as supports for enzymes in highly viscous media, such as RTILs. Moreover, SWNTs have higher specific surface area vs. MWNTs, thereby providing a greater advantage of the former for higher enzyme loading [18]. In the current work, we report the enhancement of the activity and stability and efficient biocatalyst reusability of a bacterial protease, proteinase K, when adsorbed onto SWNTs. As a result, we show the benefit of these resulting enzyme–SWNT conjugates for use as heterogeneous catalysts in RTILs.

Materials and Methods

Materials

N-acetyl-L-phenyl-alanine ethyl ester (APEE), proteinase K, and *N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide (Succ-AAPF-NA) were purchased from Sigma Aldrich (St. Louis, MO, USA) as salt-free dry powders and used without further purification. 1-Propanol,

acetonitrile, 1-butyl, 3-methylimidazolium hexafluorophosphate [BMIM (PF_6)], 1-hexyl, 3-methylimidazolium hexafluorophosphate [HMIM (PF_6)], 1-butyl, 3-methylimidazolium trifluoromethanesulfonate [BMIM (CF_3SO_3)], and 1-butyl, 3-methylimidazolium tetrafluoroborate [BMIM (BF_4)] were purchased from Sigma. 1-Octyl, 3-methylimidazolium hexafluorophosphate [OMIM (PF_6)] was purchased from Acros Organics (Morris Plains, NJ, USA). Pristine SWNTs were purchased from Carbon Nanotechnologies (Houston, TX, USA). All other chemicals were purchased from Sigma and used as obtained.

Enzyme Adsorption onto SWNTs

Proteinase K was immobilized onto SWNTs using physical adsorption as described by Karajanagi et al. [17]. Briefly, 1-mg SWNTs were sonicated in 1 ml dimethylformamide for 30 min to achieve uniform dispersion in an Eppendorf microcentrifuge tube. Dimethylformamide was then gradually replaced by 1 ml aqueous phase through repeated washing with pH 7.0 buffer (50 mM phosphate). This gradual change from organic phase to an aqueous phase renders unfunctionalized SWNTs more dispersible in water. The dispersion of SWNTs in the aqueous buffer was exposed to a freshly prepared solution of enzyme in the same buffer, and the mixture was shaken on an Innova™ 2000 (New Brunswick Scientific, Edison, NJ, USA) platform shaker for 2 h at 200 rpm and at room temperature. After incubation, the SWNTs were centrifuged at 8,000 rpm using a microcentrifuge and the supernatant was removed. Six washes were carried out, with fresh buffer added each time to remove unbound enzyme. After the washing step, all the enzyme–SWNT conjugates were pooled and 400 μl of 20 mM pH 7.8 phosphate buffer was added to enzyme–SWNT conjugates and freeze dried for 24 h.

Enzymatic Reactions

The substrate solution containing 40 mM APEE and 1 M 1-propanol was added to an RTIL in 4-ml screw-capped vials with a reaction volume of 2 ml. The reactions were initiated by adding 0.5, 2, or 5% water (v/v) to the reaction mixture. The reaction contents were shaken on an orbital shaker (200 rpm) at 50°C, and 100-μl aliquots were taken periodically for analysis. Initial rates of transesterification reactions catalyzed by proteinase K were monitored by HPLC analysis. Aliquots from the reaction mixtures were diluted fourfold with acetonitrile and analyzed on a reversed-phase C18 column (4.6×150 mm, 5 μm, Alltech Alltima, Deerfield, IL, USA). The eluent contained 0.1% (v/v) acetic acid in acetonitrile/water solution and was pumped with a linear gradient from 10 to 45% acetonitrile and then isocratically at 45% acetonitrile with a flow rate of 1 ml min^{-1}. Substrate and products were detected at 258 nm. The kinetic parameters for proteinase K-catalyzed hydrolysis in aqueous buffer were determined using the chromogenic Succ-AAPF-NA substrate [19]. The product of this reaction, *p*-nitroaniline, was followed spectrophotometrically at 405 nm and converted to molar units using the experimentally determined extinction coefficient (ε_{405}=14,250 M^{-1} cm^{-1} [16]). The values of the kinetic parameters V_{max} and K_m for the reactions in both aqueous and RTIL media were determined by fitting the reaction rates at different substrate concentrations using nonlinear Michelis–Menten fits.

Enzyme Deactivation Studies

The native enzyme and enzyme–SWNT conjugates were incubated at 70°C for 2, 4, 8, and 24 h and then cooled to room temperature. Initial rates of both enzyme preparations were

determined at room temperature and quantified by HPLC analysis. The enzyme deactivation constant was determined from the slope of the plot of ln (percent of activity retained) vs. time by fitting a straight line.

Enzyme–SWNT Reusability

Reactions using proteinase K–SWNT conjugates were performed as described earlier. After the reaction, proteinase K–SWNT conjugates were dispersed in RTIL and then centrifuged. The entire supernatant containing the substrate was then withdrawn. Proteinase K–SWNT conjugates were subsequently resuspended in fresh RTIL containing the same amount of substrate, and the reaction was repeated.

Results and Discussion

Active, stable, and reusable formulations of enzymes in RTILs are of interest to expand the repertoire of reaction media for enzymatic catalysis. As with organic solvents, many RTILs exist that encompass a diverse range of physiochemical properties. In this work, we used 3-methylimidazolium as the cationic ion platform (Fig. 1). These cations are diverse in their hydrophobic character, form stable RTILs with a number of common anions, and have been used successfully to support enzymatic catalysis [20]. Proteinase K was used as a model enzyme due to its known activity in nonaqueous media [21, 22] and its well-known thermostability [23, 24], thereby making it a good candidate for use in a range of RTILs.

Proteinase K Catalysis in RTILs

We used both hydrophobic ionic liquids [e.g., BMIM (PF_6), HMIM (PF_6), and OMIM (PF_6)] and hydrophilic ionic liquids [e.g., BMIM (BF_4) and BMIM (CF_3SO_3)] as reaction media each containing 5% (v/v) water to evaluate proteinase K catalysis. Transesterification reactions of APEE with 1-propanol were performed at 50°C and 200 rpm and the formation of both the *N*-acetyl-L-phenylalanine propyl ester and the *N*-acetyl-L-phenylalanine were followed, the latter representing potential hydrolysis due to the presence of 5% (v/v) water. Several RTILs were screened for their ability to support proteinase K catalysis (Table 1). The enzyme was lyophilized prior to use and suspended in the slightly hydrated RTILs.

Proteinase K showed the highest combined reactivity (e.g., initial rates of transesterification plus hydrolysis) in the most hydrophobic RTIL [OMIM (PF_6)] and was clearly more reactive in the water-immiscible PF_6 solvents than in the two water-miscible solvents, with reactivities up to 10-fold higher in the immiscible solvent systems than in the miscible ones. This result is consistent with previous reports for α-chymotrypsin, which has been found to be most reactive in hydrophobic water-immiscible RTILs [6].

We proceeded to investigate the reactivity of proteinase K–SWNT conjugates under identical reaction conditions as those used for the free enzyme. The loading of protein onto

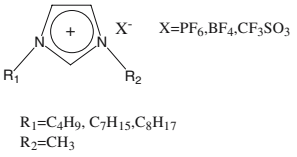

Fig. 1 Room-temperature ionic liquids with imidazolium cation changing in alkyl length and different anion

SWNTs was determined to be 0.5 mg enzyme per milligram SWNTs by spectroscopic measurements [16]. Atomic force microscopy (AFM) analysis of proteinase K–SWNT conjugates confirmed a relatively high loading of enzyme on the SWNTs (Fig. 2). The wire-like structure in Fig. 2 represents a single SWNT—a line scan in Fig. 2 for a "naked" part of the image shows a height of ca. 1.5 nm, which is consistent with a single SWNT. The globular structures on the SWNTs (ca. 6.0 nm) represent individual proteinase-K molecules. The heights of these globular structures obtained from multiple AFM images ranged from 4.5 to 8.0 nm, which is close to the size of the proteinase K (6.8×6.8×10.8 nm) [25], indicating that the enzyme likely retained its native three-dimensional structure when adsorbed onto SWNTs and was not denatured.

To confirm that the enzyme retained its near native structure, the activity of proteinase K–SWNT conjugates was determined in aqueous buffer for the hydrolysis of Succ-AAPF-NA. The V_{max}/K_m values for native enzyme and enzyme adsorbed onto SWNTs were 16.3 and 8.5 h^{-1}, respectively. Thus, the proteinase K–SWNT conjugate was ca. 50% active compared to the native solution activity, which is a relatively high value for a noncovalently attached enzyme onto a nanotube surface and attests to the retention of a native-like structure, as shown in our previous work for other enzymes [17]. The proteinase K–SWNT conjugates were also more reactive in the water-immiscible RTILs (Table 1), with the enzyme being most reactive in BMIM (PF_6). In all but one case, the enzyme–SWNT conjugates were more reactive. Based on the relatively high reactivity in BMIM (PF_6), we proceeded to investigate proteinase K reactions in this RTIL.

Effect of Water on Proteinase K Reactions in BMIM (PF6)

Because of the hydrophobic nature of BMIM (PF_6), we wanted to ensure that water contents as high as 5% (v/v) did not form a separate aqueous phase in the reaction mixture and therefore lead to a multiphasic reaction mixture that contains enzyme (or enzyme–SWNT) suspension in an aqueous-RTIL two-phase medium. Because BMIM (PF_6) is hydrophobic in nature, 5% (v/v) water is above the solubility limit in the RTIL. However, because the transesterification reactions contained nearly 7% (v/v) 1-propanol, which is miscible both in water and in BMIM (PF_6), we reasoned that the water added would be insufficient to result in a separate phase. Indeed, adding 5% (v/v) water to BMIM (PF_6) resulted in the formation of a large water droplet clearly visible in the RTIL using the aqueous buffer dye violet blue. However, upon addition of 7% (v/v) 1-propanol, this droplet disappeared and the reaction

Table 1 Total initial rates (transesterification and hydrolysis) of proteinase K–SWNT conjugates and native proteinase K for 40 mM *N*-acetyl-L-phenylalanine and 1 M 1-propanol transesterification reaction in different ionic liquids containing 5% (v/v) water.

RTIL	Initial rate (mM/mg-h)	
	PK–SWNT	Native PK
BMIM (BF_4)	4.8±0.6	1.6±0.24
BMIM (CF_3SO_3)	1.7±0.08	1.5±0.0
BMIM (PF_6)	18±2.0	10±1.0
OMIM (PF_6)	13±0.03	15±1.0
HMIM (PF_6)	13±0.3	11±0.4

PK = proteinase K

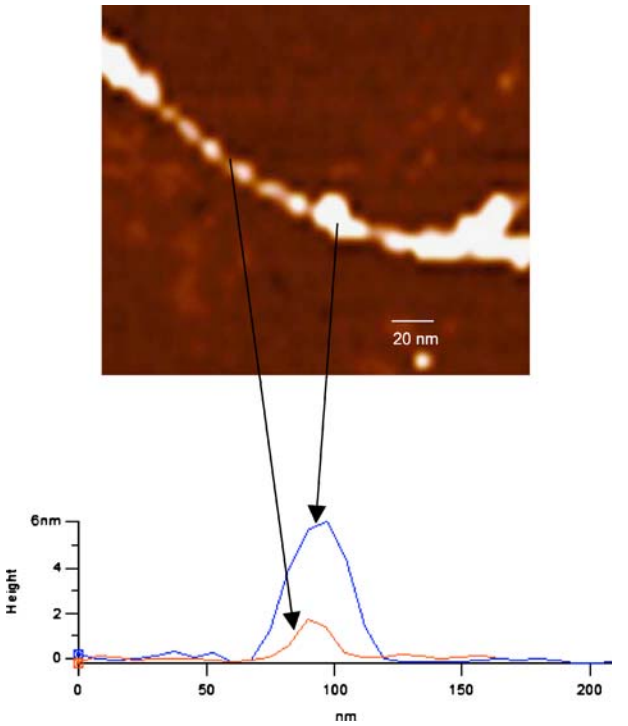

Fig. 2 Atomic force microscopy image of proteinase K adsorbed onto SWNTs. Line scans showed that a region on the SWNT without proteinase K has a height of 1.5 nm, whereas a region with proteinase K has a height of 6 nm. The difference (4.5 nm) gives the expected height of adsorbed proteinase K molecules onto SWNTs

medium was clear and homogeneous. Thus, there was no phase separation in the system; the reactions were taking place in a homogeneous system.

We evaluated the effect of water content on proteinase K catalysis in BMIM (PF_6). Identical enzyme concentrations were used (1 mg/ml) for both native enzyme and enzyme–SWNT conjugates; with a loading of 0.5 mg enzyme per milligram SWNT, this resulted in the use of 3.0 mg/ml of the conjugate. Different concentrations of water, ranging from 0.5 to 5% (v/v), were examined in BMIM (PF_6), and the initial rates for both transesterification and hydrolysis were measured. At all three water contents, the SWNT conjugates were more reactive than the free enzyme—up to nearly fivefold at both 2.0 and 0.5% (w/w) water (Table 2). This activation may be due to the greater accessibility of the enzyme to the APEE substrate when dispersed on the SWNTs or due to a relaxation in potential diffusional limitations on the SWNT conjugates due to the biocatalyst formulations being suspended in a highly viscous RTIL. We therefore proceeded to investigate in detail

Table 2 Initial rates (transesterification + hydrolysis) of proteinase K–SWNT conjugates and native proteinase K for 40 mM *N*-acetyl-L-phenylalanine and 1 M 1-propanol transesterification reaction in BMIM (PF_6).

	Total initial rate (mM/mg-h)		
	5% (v/v) water	2% (v/v) water	0.5% (v/v) water
SWNT-PK	18±2.0	7.7±0.4	2.7±0.0
Native PK	10±1.0	1.6±0.2	0.57±0.04

PK = proteinase K

potential external and intraparticle mass transfer limitations of the two proteinase K formulations in BMIM (PF_6).

Mass Transfer Considerations

Initial rates of both native and enzyme–SWNT proteinase K preparations were measured as a function of agitation speed at both 0.5 and 5.0% (v/v) added water. In no case was there a substantive rate dependence on agitation above 50 rpm (Fig. 3a, b). However, in the absence of agitation at 5% (v/v) water, evidence of external diffusional limitation was observed for both preparations. This is not surprising given the higher reactivity of the enzyme under these conditions, which pushed the reaction into at least the partially diffusion-controlled regime. To further assess the influence of external diffusion on proteinase K catalysis, we examined the reactivity of the native and enzyme–SWNT conjugate as a function of enzyme concentration. At 200 rpm, a nearly linear enzyme concentration dependence was observed for both enzyme preparations (Fig. 3c, d), which confirms essentially no external diffusional limitations upon agitation. However, in the absence of agitation, the enzymatic reaction was clearly limited by external diffusion (Fig. 3e, f). Specifically, at the highest enzyme concentration (1.5 mg/ml), the native enzyme was ca. twofold slower than expected based on linear reactivity that would be observed in the absence of diffusional limitations (Fig. 3e, dotted line). Under these conditions, slight improvement (i.e., less diffusional resistance) was obtained with the enzyme–SWNT conjugates (Fig. 3f).

In addition to external diffusional limitations, the highly viscous ionic liquid could also impart intraparticle diffusional limitations to the native enzyme suspension, whereas the surface-bound enzyme–SWNT conjugates would be expected to be unaffected by intraparticle diffusional effects, as the enzyme is bound to the surface. To assess the influence of intraparticle diffusion on the native enzyme suspension in RTILs, we calculated the observable modulus (Φ) for both spherical particles (Eq. 1) [26].

$$\Phi = \frac{v_{\mathrm{obs}}}{D_{\mathrm{eff}} S_{\mathrm{o}}} \left(\frac{R}{3}\right)^2 \tag{1}$$

$$D = \frac{k_{\mathrm{B}} T}{6\pi\mu r} \tag{2}$$

Here, v_{obs} is the observed reaction rate per volume of catalyst and D_{eff} is the effective diffusivity of the substrate obtained from the Stokes–Einstein relationship (Eq. 2). The particle size was estimated via light microscopy (of a sample removed from the reaction mixture) to be approximately 1.2 and 1.3 μm at 5.0% (v/v) and 0.5% (v/v) water, respectively. Thus, for the native proteinase K suspension, the values of Φ were calculated to be 11 and 2.0 at 5.0 and 0.5% (v/v) water, respectively. Hence, the native enzyme was subject to substantial intraparticle diffusional limitations with an effectiveness factor under 0.1.

Apparent Enzyme Kinetics

Because external diffusional limitations could be eliminated by agitation, we proceeded to investigate the apparent kinetics of the proteinase K–SWNT conjugates in BMIM (PF_6). Apparent values of $V_{\max}$ and K_{m}, as well as the catalytic efficiency ($V_{\max}/K_{\mathrm{m}}$), were

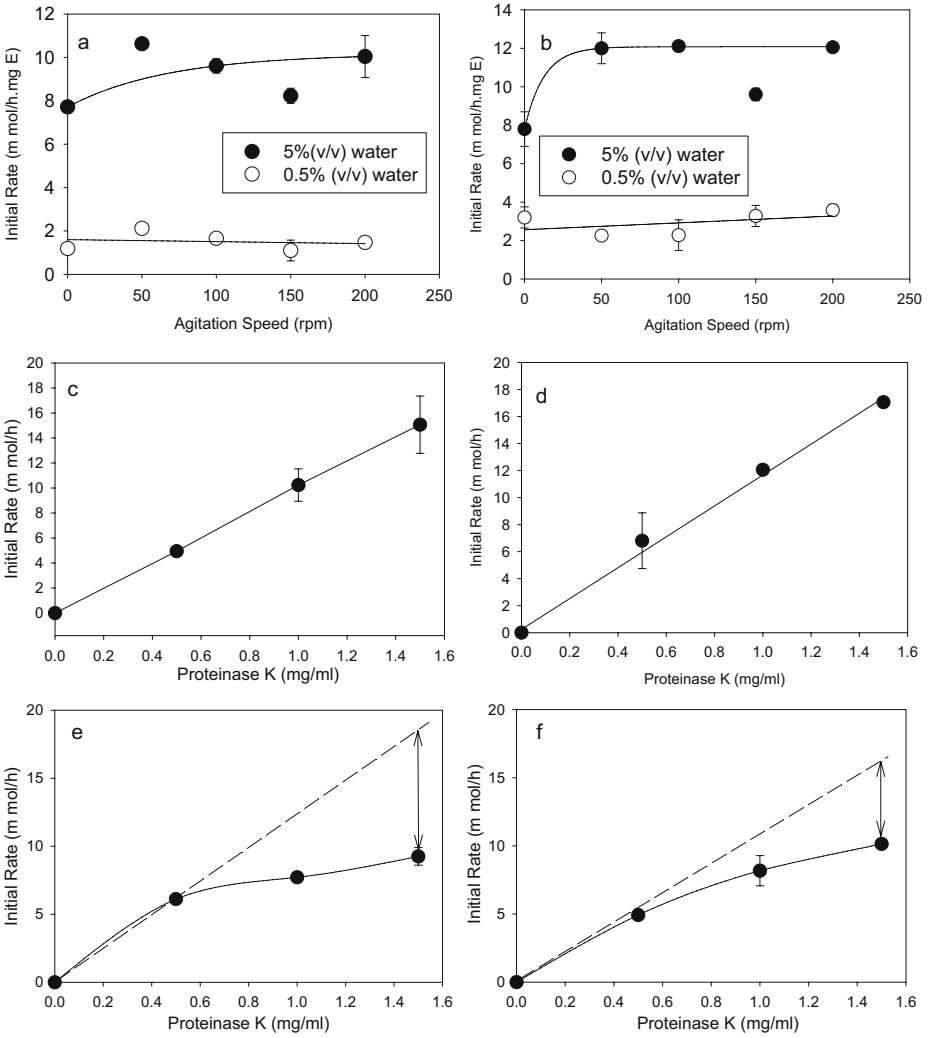

Fig. 3 **a** Total initial rates (transesterification plus hydrolysis) as a function of agitation rate for native proteinase K (1 mg in 1 ml reaction volumes) at 5.0% (v/v) water (*closed circles*) and at 0.5% (v/v) water (*open circles*). **b** Total initial rates as a function of agitation rate for proteinase K–SWNT (1 mg/ml enzyme) at 5.0% (v/v) water (*closed circles*) and at 0.5% (v/v) water (*open circles*). **c** Total initial rates (transesterification and hydrolysis) as a function of enzyme concentration for native proteinase K at 5% (v/v) water and 200 rpm agitation. **d** Total initial rates as a function of enzyme concentration for proteinase K–SWNT conjugates at 5.0% (v/v) water and 200 rpm agitation. Effect of enzyme loading of native proteinase K at 5.0% (v/v) water under stagnant conditions and expected total initial rate profile based on the situation without external diffusional limitations for native enzyme (**e**) and proteinase K–SWNT conjugates (**f**)

determined as a function of water content. As summarized in Table 3, proteinase K–SWNT conjugates showed dramatically higher catalytic turnover (combination of transesterification and hydrolysis) in the RTIL at all three water concentrations when compared with the hydrolysis reaction in aqueous buffer—in the case of 5% (v/v) water, the V_{max} in the RTIL was ca. 13-fold higher than that in water. This increased catalytic turnover in RTILs relative

Table 3 Kinetic parameters of total reaction (transesterification and hydrolysis) catalyzed by proteinase K–SWNT and native proteinase K conjugates in BMIM (PF_6).

Enzyme	Kinetic parameters				
		Aqueous buffer	5.0% (v/v)	2.0% (v/v)	0.5% (v/v)
PK–SWNT	V_{max}/K_m (h^{-1})	8.5±1.62	0.39±0.10	0.28±0.05	0.08±0.02
	V_{max} (mM mg^{-1} h^{-1})	4.1±0.41	54±5.4	25±1.1	11±0.7
	K_m (mM)	0.03±0.003	140±37	89±11	130±22
Native PK	V_{max}/K_m (h^{-1})	16.3±2.28	0.19±0.06	0.05±0.01	0.02±0.01
	V_{max} (mM mg^{-1} h^{-1})	6.4±0.34	8.4±0.60	7.8±0.80	0.37±0.03
	K_m (mM)	0.02±0.002	43±11	160±31	20±3.4

PK = proteinase K

to aqueous buffer does not necessarily indicate that the intrinsic reactivity of the enzyme is higher in the RTIL, as the reaction in RTIL represents a mixed kinetic profile consisting of both transesterification and hydrolysis reactions. Nevertheless, the high apparent V_{max} in the RTIL strongly suggests that the intrinsic catalytic turnover of proteinase K–SWNT conjugates is not substantially altered by the combination of adsorption of the enzyme onto SWNTs and placing the conjugates into the RTIL. The RTIL, however, caused a dramatic increase in the substrate K_m value. This is not surprising given the very high solubility of APEE in the RTIL as compared to water. As a result, the substrate ground state is dramatically stabilized in the RTIL, as opposed to water, and this results in a substantial increase in apparent K_m, similar to that observed for subtilisin catalysis in organic solvents [27].

The native enzyme showed lower catalytic efficiency than the enzyme–SWNT conjugate. Some of this is partly due to the presence of intraparticle diffusional limitations. However, some may be due to a poor accessibility of the enzyme within the micron-sized enzyme particles. Nonetheless, even the native enzyme showed higher catalytic turnover than compared with soluble enzyme in aqueous buffer. Thus, proteinase K showed high catalytic activity in the RTIL.

Enzyme Processibility

Enzyme denaturation and enzyme reuse are of significant importance for ultimate commercial application. Therefore, enzyme thermostability and reusability was investigated for both the enzyme–SWNT conjugates and the native enzyme preparation to test enzyme processibility in RTILs. The proteinase K–SWNT conjugates showed enhanced thermostability, as reflected in terms of higher optimal temperature (T_{opt}) and enzyme half life ($t_{1/2}$). Upon conjugation of proteinase K onto SWNTs, T_{opt} is enhanced 10 and at least 40°C at 5.0 and 2.0% (v/v) water, respectively, compared to the native enzyme (Fig. 4a, b). The thermal stabilization of the enzyme–SWNT conjugate is accompanied by stabilization against irreversible denaturation. Specifically, the $t_{1/2}$ of proteinase K was enhanced twofold by adsorption onto SWNTs at 5% water ($t_{1/2}$=14 and 6.2 h for enzyme–SWNT conjugates and native enzyme, respectively) and nearly fivefold at 2% water ($t_{1/2}$=59 and 13 h for enzyme–SWNT conjugates and native enzyme, respectively). The increased stabilization of enzyme upon conjugation onto the SWNTs is likely a result of immobilization-induced stabilization, which is well known for conventional supports [28].

In addition to the observed stabilization of proteinase K due to adsorption onto SWNTs, the ease of nanotube filtering enabled the facile reuse of the proteinase K–SWNT conjugates.

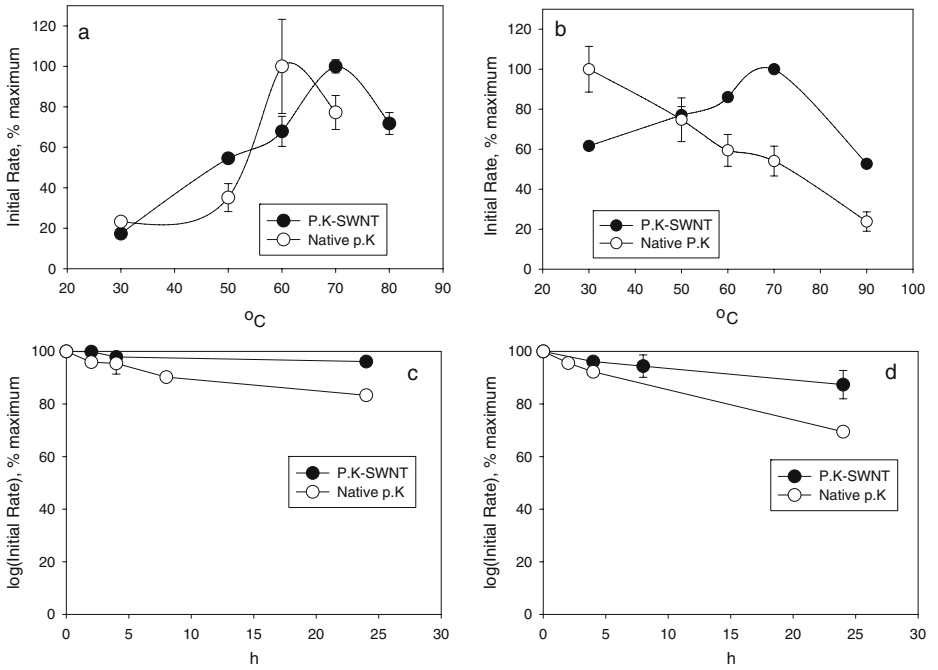

Fig. 4 Normalized total initial rate (transesterification plus hydrolysis) as a function of reaction temperature at 2% (v/v) water (**a**) and 5% (v/v) water (**b**) for both native proteinase K (*open circles*) and proteinase K–SWNT conjugates (*closed circles*). Residual activity of proteinase K following incubation at 70°C for reactions in 2% (v/v) water (**c**) and 5% (v/v) water (**d**). The enzyme concentration in all cases was 1 mg/ml

This reusability was tested for both the native and enzyme–SWNT conjugates at 5.0% (v/v) water. Because there was some enzyme–SWNT conjugate loss in each run of reusability experiments, we measured the specific activity by normalizing the total initial rate per milligram enzyme. The amount of enzyme at the end of the fifth run was obtained by filtration of the enzyme–SWNT conjugates through a 0.8-μm polycarbonate membrane filter and overnight drying. The amount of enzyme per run was then calculated by assuming a

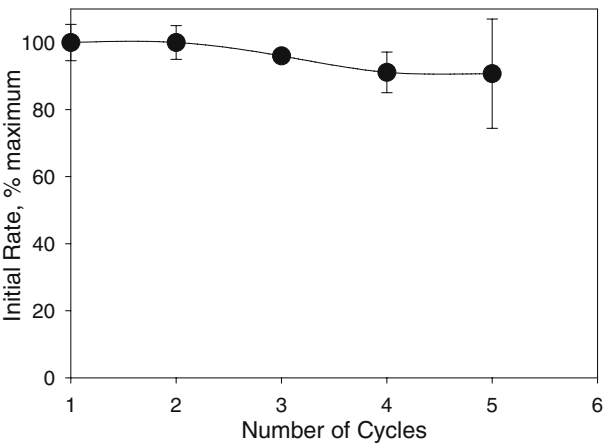

Fig. 5 Reusability of proteinase K–SWNT conjugates. See text for experimental details

linear enzyme loss between the first and fifth runs of reusability experiments. Proteinase K–SWNT conjugates retained most of their specific activity at 5% (v/v) water in BMIM (PF_6) (Fig. 5), whereas native proteinase K was inactive after one use at the same water content.

In conclusion, we have demonstrated that proteinase K–SWNT conjugates are highly dispersed, even in viscous RTILs, and are far less diffusionally limited than native enzyme suspensions. In aqueous solutions, the enzyme–SWNT conjugates are ca. 50% as active as the native solution counterpart and, even in the RTILs, the activity of the enzyme–SWNT conjugate remains high. Apparent enzyme kinetic investigations revealed that the catalytic efficiency (V_{max}/K_m) of proteinase K–SWNT conjugates in RTILs is strongly influenced by the poor substrate partitioning into the enzyme's active site (e.g., high apparent K_m values), with apparent values of catalytic turnover (V_{max}) significantly exceeding that in aqueous solutions. Proteinase K–SWNT conjugates provide highly active, thermally stable, and reusable biocatalyst preparations in RTILs. Efforts are currently underway to explore other enzyme–carbon nanotube conjugates in RTILs to establish the general nature of the enhanced reactivity, stability, and processibility of these unique enzyme formulations.

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