Enzyme-Catalyzed Hydrolysis of Cellulose in Ionic Liquids: A Green Approach Toward the Production of Biofuels

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We investigated the reactivity and stability of a commercial mixture of cellulases in eight ionic liquids by optical and calorimetric techniques. First, hydrolysis by cellulases from *Tricoderma reesei* in these ionic liquids was benchmarked against that in aqueous buffer. Only 1-methylimidazolium chloride (mim Cl) and tris-(2-hydroxyethyl)methylammonium methylsulfate (HEMA) provided a medium in which hydrolysis could occur. While hydrolysis at 65 °C is initially much faster in buffer than in these two liquids, it reaches a plateau after 2 h, whereas the reaction progresses monotonically in the two ionic liquids. This difference in the rate of hydrolysis is largely attributed to two factors: (1) the higher viscosity of the ionic liquids and (2) the enzymes are irreversibly denatured at 50 °C in buffer while they are stable to temperatures as high as 115 °C in HEMA. We explored whether fluorescence quenching of aromatic amino acids of the enzymes was indeed a signature of protein denaturation, as has been suggested in the literature, and concluded that quenching is not necessarily associated with denaturation. When it does occur, for example, in the presence of ionic liquids formed from imidazolium cations and chloride anions, it arises from the imidazolium rather than the chloride. Finally, we conclude that HEMA is a promising, novel, green medium for performing cellulose hydrolysis reactions to convert biomass into biofuels. Because of the thermal stability it imparts to enzymes, its ability to solubilize biomass, and the fact that it does not quench tryptophyl fluorescence (thus permitting monitoring of the enzymes by fluorescence spectroscopy), HEMA provides an ideal starting point for the design of ionic liquids, not only for the hydrolysis of biomass, but also for use with a wide spectrum of enzymatic reactions.

Introduction

Converting biomass into fuel is becoming increasingly important owing to the desirability of finding substitutes for fossil fuels and to the need to address the problem of global warming. Cellulose, one of the main constituents of biomass, is the most abundant biorenewable material on the planet. Consequently, during the past two decades, considerable effort has been devoted to the hydrolysis of cellulose in order to convert it into fuel.[1−7] There are, however, limitations to this process that are imposed mainly because of the limited solubility of cellulose in water or other organic solvents. That is, cellulose is a linear polysaccharide chain (Figure 1a) consisting of hundreds to thousands of D-anhydroglucopyranose linked together by $\beta(1\rightarrow4)$-glycosidic bonds;[3] and this highly symmetrical polymer is sparingly soluble in most solvents. Thus the traditional dissolution processes are not only cumbersome and expensive, but they also require extreme conditions,[8] which in turn may cause serious environmental problems because the solvents they require, such as LiCl/*N*,*N*-dimethylacetamide, *N*-methylmorpholine *N*-oxide/water, DMSO/paraformaldehyde, etc., are not only volatile, toxic, and costly; but they also cannot be recovered and reused.[9,10]

Recently, it has been reported that a special class of solvents, room temperature ionic liquids (RTILs), can dissolve cellulose.[9,11−19] Ionic liquids are green solvents, in contrast to volatile organic compounds (VOCs), owing to their high chemical and thermal stabilities, negligible vapor pressure,[20] and high recoverability and reusability.[21,22] These properties have piqued the interest of the scientific community, and a variety of fundamental studies[23−29] have been performed on them to obtain a better understanding of their characteristics. Rogers and coworkers[9,15−17,30] have performed extensive studies on the dis-

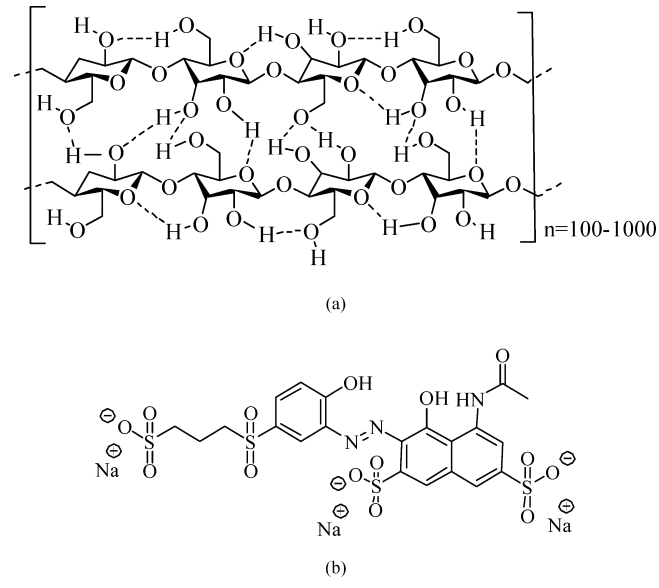

Figure 1. Structure of (a) the linear polymer chain of cellulose, showing inter-chain hydrogen bonding interactions, and (b) remazol brilliant violet dye tagged to the cellulose chain in cellulose azure.

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solution of cellulosic materials in different ionic liquids. They have shown that ionic liquids can be used as nonderivatizing solvents for cellulose. Among the different solvents they studied, 1-butyl-3-methylimidazolium chloride (bmim Cl) was found to be most effective in dissolving cellulose, and they attributed this effect to strong hydrogen bonding interactions of the hydroxyl group with the halide anion.[9,15,30] They have also reported the dissolution of other lignocellulosic sources, such as wood[17] and banana pulp,[16] in ionic liquids. Sheldon and co-workers have shown high solubility of di- and polysaccharides in ionic liquids containing dicyanamide anions.[12] More recently it has been found that *Bombyx mori* silk, fibroin,[11] and hard and soft woods[18] are readily solubilized in imidazolium based ionic liquids.

Of the several steps involved in the production of ethanol from cellulose, the most crucial and difficult is the cellulolysis, which is the hydrolysis of the cellulose polymer chain into glucose units.[3,5,7] Different catalysts have been used for this reaction, such as metal chlorides,[4,6] acids, or enzymes.[1,31,32] The most common and widely used enzyme for this saccharification of cellulose is cellulase. To make the entire process of enzyme-catalyzed hydrolysis of cellulose green, the use of ionic liquids as solvents or cosolvents has received growing attention. The most thoroughly studied enzyme in ionic liquids is *Candida antarctica* lipase B, used to catalyze transesterification reactions.[31,33,34] While the hydrophobic effect that increases protein stabilization is absent in organic ionic liquids, one advantage to biocatalysis in ILs as opposed to aqueous buffers is the longer activity of enzymes in ILs, which is thought to arise from the slow breaking and remaking of hydrogen bonds in the non-aqueous medium.[35] Studies of cellulase-induced catalysis in ionic liquids are, however, still limited.[5,36−39]

The physical and chemical properties of ionic liquids vary considerably depending on their cation−anion pair. Several attempts have been made to explore the activity of enzymes in ionic liquids, and there are various issues concerning the stability of these biomacromolecules in ionic liquids. Most of them are ineffective for biocatalyis. It has been suggested that ionic liquids containing the anions Cl^-, Br^-, NO_3^-, and $CF_3SO_3^-$ denature enzymes owing to their higher basicity and, hence, higher affinity for hydrogen bonds.[22,40] There are diverse opinions concerning the effect of fluorinated anions, such as BF_4 and PF_6, on the enzyme's lifetime. Some reports suggest that since charge can be distributed over several fluorine atoms, the hydrogen bond affinity is minimized between the solvent and the enzyme and that, consequently, there is no interference with the internal hydrogen bonding network of the enzyme, maintaining its secondary structure.[31] On the other hand, Swatloski et al.[41] have reported that BF_4 and PF_6 have a high propensity to undergo self-decomposition with the liberation of HF, which is detrimental for the enzymes.

One must also consider the trade-off between viscosity and solubility. The inherent high viscosity of the ionic liquids is a retarding factor for the rate of enzymatic hydrolysis, since it slows the diffusion of the enzyme to its target. Viscosity increases with the length of the alkyl chain. Although the highly viscous bmim Cl (Table 1) slows down the rate of cellulase induced hydrolysis of cellulose,[36] it can dissolve a substantial amount of cellulose.[9] As opposed to bmim Cl, cellulose has very limited solubility in bmim PF_6 and bmim BF_4, whereas the latter keeps the enzyme active. Thus it is very challenging to find the right combination for the cation−anion pair that can form a compromise between the opposing factors of dissolving cellulose, retaining the activity of the enzymes, and having a low viscosity.

TABLE 1: Viscosity of Various Solvents

solvent	temp (°C)	η (cP)[a]
H_2O[55]	20	1.0
	50	0.55
	65	0.43
bmim Cl[56]	30	11000
	70	330
mim Cl	20	9.8 ± 0.2
	30	6.8 ± 0.1
	50	4.2 ± 0.1
	65	3.0 ± 0.1
	85	2.2 ± 0.1
HEMA	20	1460 ± 30
	30	640 ± 8
	50	230 ± 5
	65	110 ± 2
	85	50 ± 1

[a] Viscosity in centipoise for various solvents, including the ionic liquids: 1-butyl-3-methylimidazolium chloride (bmim Cl); 1-methylimidazolium chloride (mim Cl); and tris(2-hydroxyethyl)-methylammonium methylsulfate (HEMA).

Consequently, this work delves into the activity of cellulase in eight ionic liquids and is motivated by that of Rogers and co-workers, who studied the activity of cellulase from *Trichoderma reesei* in bmim Cl and bmim BF_4.[36] They reported that the enzymatic activity ceases within an hour of reaction due the presence of a high concentration of chloride ions in the bmim Cl, whereas the activity is retained in bmim BF_4. Enzymatic activity was monitored as a function of time with cellulose azure (an azo-dye tagged to the cellulose chain),[42] which upon hydrolysis releases the dye, whose absorbance is measured as a signature of the progress of reaction. In this work, we employ steady-state optical absorbance and fluorescence measurements as well as differential scanning calorimetry and thermal and microwave heating techniques to understand the stability of cellulase and its activity in different ionic liquids. We found that certain ionic liquids stabilize the cellulases at temperatures as high as 115 °C whereas the enzymes are irreversibly denatured at 50 °C in aqueous buffer. Hydrolysis in ionic liquids is slower than that in buffer, which is attributed to the higher viscosity of the ionic liquids. Furthermore, while quenching of the fluorescence of the intrinsic amino acids of cellulases has been interpreted as a signal of protein denaturation (attributed to chloride ions), we demonstrate that such quenching is not necessarily associated with denaturation. When it does occur, for example, in the presence of ionic liquids formed from imidazolium cations and chloride anions, it arises from the imidazolium rather than the chloride.

Experimental Section

Materials and Methods. GC 220 cellulase from *Tricoderma reesei* (1.2 g/mL) was a gift from Genecor International Co. and was used without further purification. GC 220 is a heterogeneous mixture of several cellulase components, which includes endoglucanases and cellobiohydrolases. In the subsequent discussion, whenever we refer to "cellulase" or "the enzyme," we are referring globally to the GC 220 preparation. Cellulose azure from Sigma Aldrich, which although may not be used for quantitative purposes is appropriate for comparative activity studies,[43] was washed multiple times with deionized water to eliminate any unbound dye particles from the surface of cellulose, and was dried before use. (It is made from purified

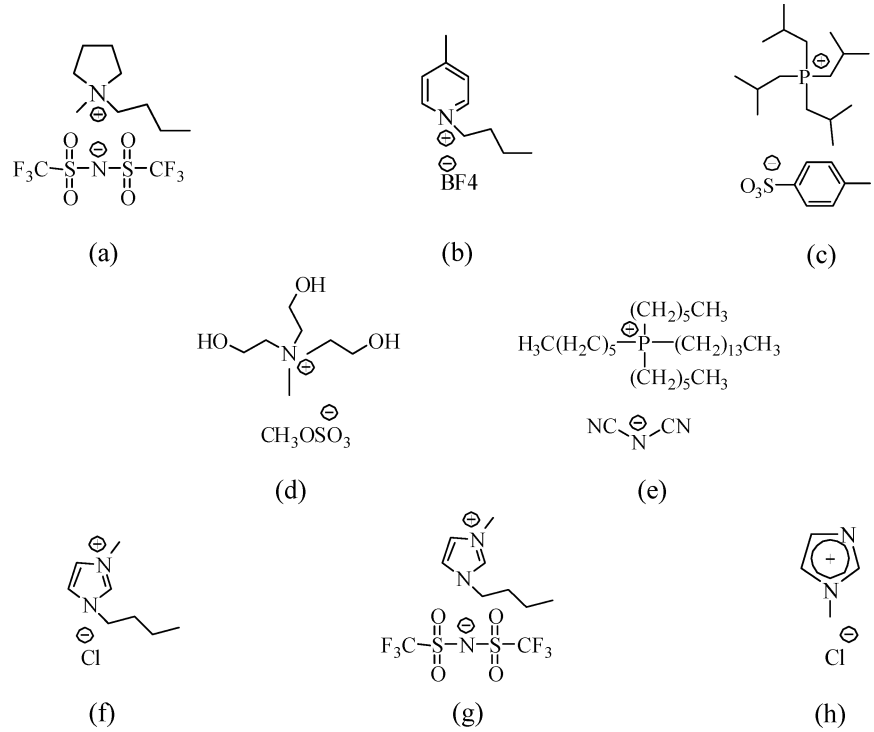

Figure 2. Structures of ionic liquids studied: (a) bmpyro NTf_2, (b) bmpy BF_4, (c) bmph OTs, (d) HEMA, (e) hdph dca, (f) bmim Cl, (g) bmim NTf_2, and (h) mim Cl. See the text for a definition of the abbreviations.

cotton linters and then covalently tagged with remazol brilliant violet dye (Figure 1b).) Upon hydrolysis of cellulose, the dye is solubilized and its absorbance reports on the activity of the enzyme. The ionic liquids used in this study are given in Figure 2. 1-Butyl-1-methylpyrrolidinium bis(trifluoromethane)sulfonimide (bmpyro NTf_2), 1-butyl-4-methylpyridinium tetrafluoroborate (bmpy BF_4), triisobutylmethylphosphonium tosylate (bmph OTs), tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA), trihexyltetradecylphosphonium dicyanamide (hdph dca), and BASF grade 1-butyl-3-methylimidazolium chloride (bmim Cl) were purchased from Sigma Aldrich. 1-Methylimidazolium chloride (mim Cl) and 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (bmim NTf_2), were synthesized as described elsewhere.[23] Ionic liquids were not dried before use, since aqueous enzyme solution was introduced into them before performing the experiments. Viscosity measurements were made with a ViscoLab 4000 piston style viscometer from Cambridge Applied system at the desired temperatures.

Steady State Measurements. Steady-state absorption spectra were obtained on a Varian Cary 100 Bio UV−visible spectrophotometer with 1-nm resolution equipped with a Peltier temperature controller. All the activity measurements were done in a double-beam spectrometer, where both sample and reference cells were maintained at identical conditions. The latter contained substrate without enzyme for automatic subtraction of the absorbance of the liberated dye (if any) in the absence of enzyme. The concentration of cellulose azure was 1.5 mg/mL, and that of cellulase, 0.7 mg/mL. Steady-state fluorescence spectra were obtained on a Spex Fluoromax-4 with a 2-nm bandpass and corrected for lamp spectral intensity and detector response. For both fluorescence and absorption measurements, a 1-cm path length quartz cuvette was used. All cellulase samples were excited at either 284 or 295 nm and identical spectra were obtained at both wavelengths. In all the solvents, the enzyme was equilibrated for at least 1 h with constant stirring at the desired temperature before the fluorescence measurements were done.

Differential Scanning Calorimetry Measurements. Differential heat flow curves were measured with a Q 10 Differential Scanning Calorimeter (TA Instruments), attached to a liquid nitrogen cooling system (LNCS). The sample and reference pans were filled with 70 μL of the solutions. Cellulase in pH 4.8 citrate buffer and cellulase in ionic liquids were scanned from 10 to 100 °C and from 10 to 150 °C, respectively. The enzyme concentration was maintained at 2.13−4.5 mg/mL. DSC scans were also done with pure buffer and ionic liquids without enzymes to ensure that no transitions occurred in the same temperature range.

Results and Discussion

Optical Measurements Probing Enzymatic Stability and Function. Steady-state fluorescence from cellulase (GC 220 obtained from the fermentation of the fungus *T. reesei*) was monitored in eight ionic liquids at room temperature and compared with that obtained in pH 4.8 citrate buffer (Figure 3). GC 220 consists of a mixture of enzymes. In each of the enzymes, there are several tryptophans, which provide intrinsic fluorescent markers. Misfolding or unfolding of the enzyme is typically accompanied by quenching of fluorescence or a red shift[44] of the emission spectra. Baker et al. have shown that thermal denaturation of four *T. reesei* components was accompanied by quenching of fluorescence and a red shift in the emission maxima.[45] All the ionic liquids, except HEMA, quenched nearly all of the cellulase fluorescence. Interestingly, cellulase showed higher fluorescence in HEMA than in buffer, with retention of spectral shape. This is consistent with higher stability of the enzyme in the ionic liquid at ambient conditions, but it is not a necessary condition for stability as we shall see below.

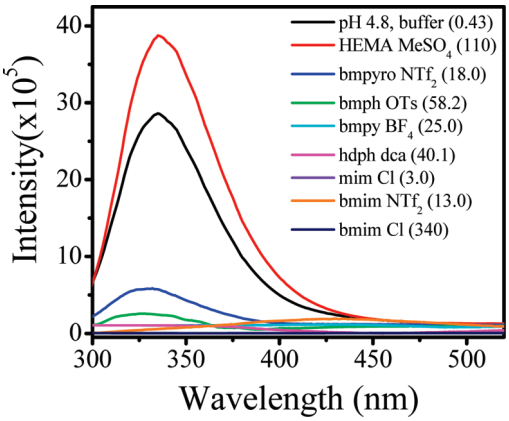

Figure 3. Steady-state emission spectra of cellulase (2.13 mg/mL) in pH 4.8 buffer and ionic liquids at room temperature. The samples were excited at 284 nm. Enzyme fluorescence is quenched in all the ionic liquids except for HEMA, which shows higher fluorescence than in buffer, with retention of spectral shape and peak maxima. The numbers in parentheses are the viscosities measured in centipoise (cP) at 65 °C. The spectrum of cellulase in mim Cl is not visible due to superposition with other spectra.

The activity of the enzyme was monitored at 65 °C in pH 4.8 buffer and in all eight ionic liquids with cellulose azure as the substrate. The absorbance of the solubilized dye increased with time in pH 4.8 buffer, mim Cl, and HEMA (Figure 4), but the initial rate of hydrolysis was much faster in buffer. After 1 h, the absorbance due to the liberated dye became constant, whereas it increased monotonically for the two ionic liquids, mim Cl and HEMA. The plateau observed in the absorbance curve for the buffer is not due to the exhaustion of the substrate in the reaction medium, since visibly detectable amounts of unreacted solid cellulose azure remained in the reaction vessel in all of the activity experiments. Because cellulase in buffer denatures, its activity ceases; and no further hydrolysis occurred. On the other hand cellulase remains active and stable in mim Cl and HEMA, and hydrolysis can continue. Cellulase did not show any activity in the other ionic liquids.

Rogers and co-workers[36] have argued that bmim Cl deactivates the enzyme within an hour due to the presence of a high concentration of chloride ions and interpret the quenching of trytophan fluorescence in that ionic liquid as a signature of denaturation. We found that the tryptophan fluorescence is completely quenched in mim Cl, which is consistent with the reports of Rogers and co-workers for bmim Cl; but the hydrolysis of cellulose continued even 1 h after the addition of cellulase in mim Cl. Negligible hydrolysis of cellulose was observed in the absence of enzyme in mim Cl, contrary to the observation of Vanoye and co-workers, who reported that, owing to its inherent acidity, mim Cl can act as both a catalyst and solvent in the dehydration of fructose.[46] The difference between our work and that of Vanoye and co-workers merits comment. The latter reports on the dehydration of simple sugar molecules, such as fructose and sucrose, to yield 5-hydroxymethylfurfural (HMF) using the ionic liquid 1-methylimidazolium chloride (mim Cl), in which the latter acts as both a catalyst and solvent. Dehydration of sugars is reported to be catalyzed by acids.[6] Owing to the inherent Brønsted acidity of mim Cl, the dehydration process is facilitated as shown in their work. Our experiments, on the other hand, deal with a much more complicated polymer, cellulose, as opposed to monomeric sugar units such as glucose, fructose, etc. Cellulose (Figure 1a), as noted earlier, is a polydispersed linear homopolymer of β-(1→4)-glycosidic linked D-anhydroglucopyranose units, with intra- and

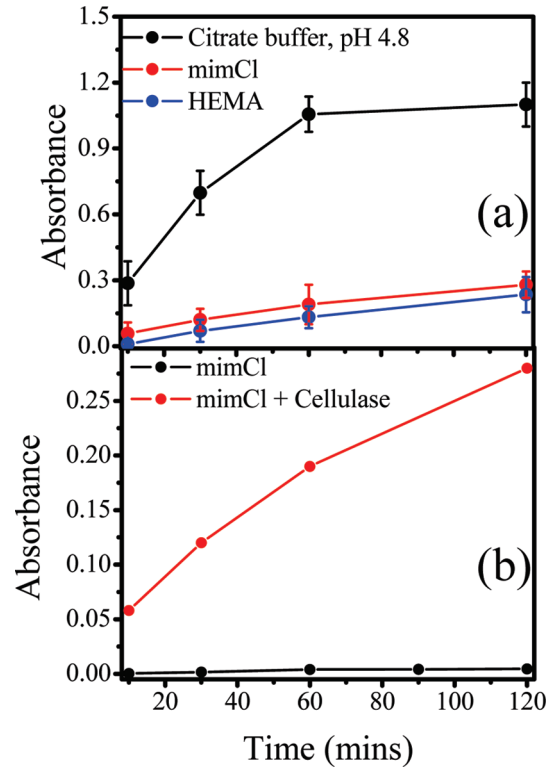

Figure 4. (a) Cellulase activity at 65 °C as monitored via the absorbance of cellulose azure (1.5 mg/mL) at 575 nm in pH 4.8 citrate buffer, mim Cl, and HEMA. In buffer, cellulase showed maximum activity, which is represented by higher absorbance of the liberated dye, compared to those in the ionic liquids. We attribute this to the relative viscosities of the solvents. On the other hand, in buffer there is almost no hydrolysis after 2 h (i.e., the absorbance reaches a plateau), whereas the reaction progresses monotonically in the ionic liquids. (b) Hydrolysis of cellulose azure (1.5 mg/mL) monitored as a function of time, as in panel a, in mim Cl with and without cellulase. The red data points correspond to the absorbance of dye liberated only due to hydrolysis by cellulase, whereas almost negligible hydrolysis (black dots) was observed without the enzyme.

inter-chain hydrogen bonds. Hydrolysis of cellulosic polymer is much more complicated than dehydration of a single sugar unit. This is most likely the reason for mim Cl being unable to cause substantial hydrolysis of cellulose (Figure 4b).

Furthermore, we monitored the fluorescence intensity of tryptophan in a buffer as a function of Cl^- and did not observe any quenching even at 1 M NaCl. At 6 M NaCl (the highest concentration obtainable in water at room temperature), only slight quenching was observed (Figure 5). On the other hand, efficient quenching was found on the addition of the cationic moiety of the ionic liquid, 1-methylimidazole (Figure 5). A Stern−Volmer quenching plot was biphasic, with $K_{sv} = 3.8\ M^{-1}$ up to 0.4 M and with $K_{sv} = 89\ M^{-1}$ from 0.4 to 1 M, which is consistent with the trends reported by Engelborghs and co-workers.[47,48] The efficiency of quenching increases in the presence of positively charged imidazolium cation, due to electron transfer from tryptophan to the imidazolium ring. Thus the quenching of fluorescence from cellulase is not due to chloride ions, but to the imidazolium moiety.

Enzymatic Stability: Calorimetry and Temperature Studies. To characterize the behavior and stability of cellulase in the ionic liquids, temperature-induced unfolding of the enzymes was studied with optical absorbance and differential scanning calorimetry measurements. The stability of cellulase in buffer, mim Cl, and HEMA was monitored by recording the absorbance

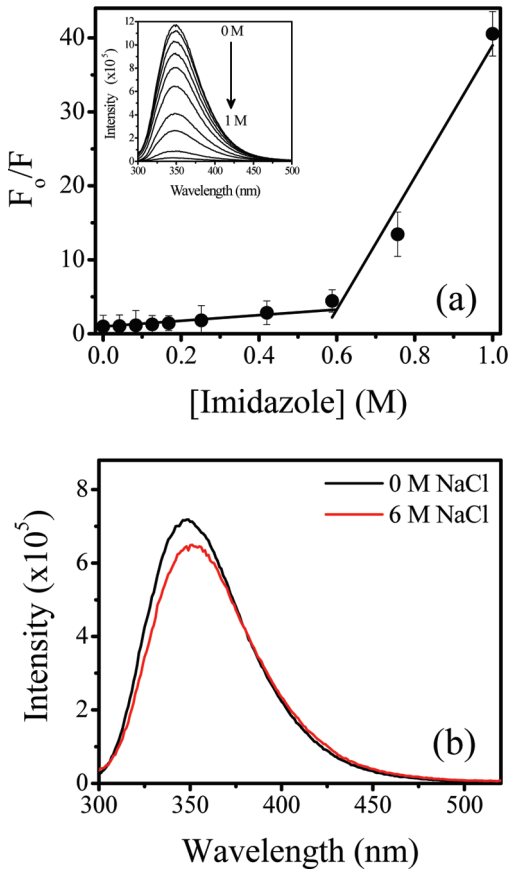

Figure 5. Stern−Volmer quenching plot of tryptophan in pH 7.0 buffer with 1-methylimidazole, where F and F_0 are the fluorescence intensities with and without quencher. The samples were excited at 284 nm. The inset shows a decrease of the fluorescence intensity of tryptophan with increasing quencher concentration, indicated by the downward arrow. The data points were fit to two straight lines to obtain the Stern−Volmer quenching constants (K_{sv}), which were found be 3.8 and 89 M^{-1}. (b) Representative fluorescence spectra of tryptophan (6×10^{-7} M) in pH 7.0 buffer and aqueous 6 M NaCl solution. The quenching of fluorescence due to chloride ions is negligible compared to that of 1-methylimidazole. This demonstrates that complete loss of fluorescence from cellulase in mim Cl is due to the imidazolium cation rather than to the chloride anion. For purposes of comparison, pure mim Cl is 9 M in chloride ion.

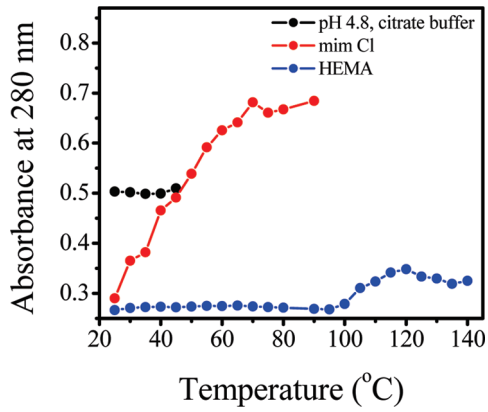

Figure 6. Temperature dependence of absorbance of trytophan in cellulase in buffer, mim Cl, and HEMA. The cellulase precipitates at 50 °C in buffer, indicating denaturation of the enzyme and limiting the temperature range for the experiment to 45 °C. The transition from the native to the unfolded state was observed at ∼110 °C and is associated with a sudden increase in the absorbance of the enzyme in HEMA. No such denaturation profile was observed in mim Cl.

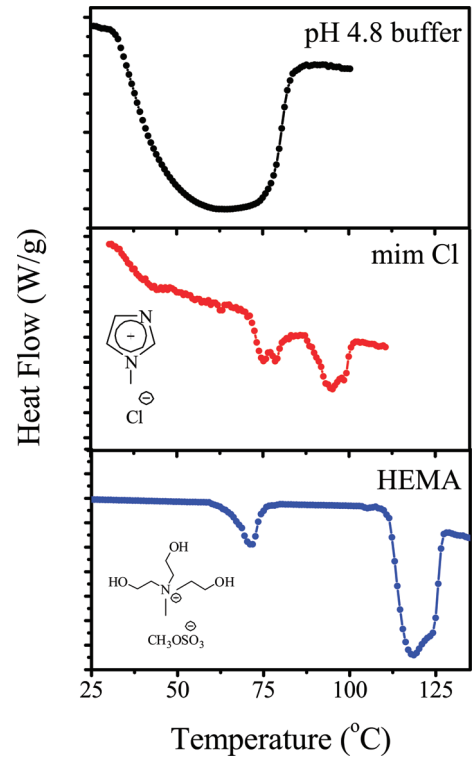

Figure 7. DSC heat-flow profiles of cellulase in pH 4.8 citrate buffer (black), mim Cl (red), and HEMA (blue). Broad transition peaks were obtained due to the presence of multiple cellulase components from *T. reesei* fungus. The transition temperature ($T_{1/2}$) in buffer at ∼60−75 °C, and in ionic liquids at 74−85 °C for mim Cl and 115−125 °C for HEMA, shows that the ionic liquid imparts higher heat resistance to cellulase enzymes, indicating greater thermal stability.

of tryptophan at 280 nm as a function of temperature (Figure 6). Conformational changes have been associated with large absorbance changes as a function of temperature.[49] The buffer solution containing the enzyme turned turbid at 50 °C, thus absorbance was recorded until 45 °C. In both ionic liquids, however, no turbidity was observed. A jump in absorbance was found at temperatures greater than 100 °C in HEMA, which probably is an indication of a conformational change in the cellulase, whereas in mim Cl, a monotonic increase of absorbance was observed with temperature. Reversibility of the unfolding of cellulase in the ionic liquid was observed by slow cooling, which led to a drop in the absorbance to the initial value in HEMA which was not observed in mim Cl or in buffer. The precipitated enzyme in buffer did not dissolve on cooling. Thus the cellulase is probably more stable and gains higher heat resistance in the HEMA than in buffer.

Heat flow curves from differential scanning calorimetry (DSC) measurements in Figure 7 show thermal unfolding of cellulase in pH 4.8 citrate buffer, mim Cl, and HEMA. The minima in the heat flow curves correspond to $T_{1/2}$, the "transition temperature" where 50% of the enzyme is unfolded. The transition peaks are very broad because of the presence of multiple enzyme components from *T. reesei*. Baker et al.[45] reported DSC studies of four major enzyme components produced *T. reesei,* which are the two endoglucanases, EG I and EG II, and the two cellobiohydrolases, CBH I and CBH II. CBH I, CBH II, and EG I have nearly identical transition temperatures at ∼64 °C, whereas the EG II shows a transition temperature at ∼75 °C in pH 4.8 acetate buffer. Our results showed a broad transition peak ranging from 60 to 75 °C in pH 4.8 citrate buffer, which is in good agreement with peaks

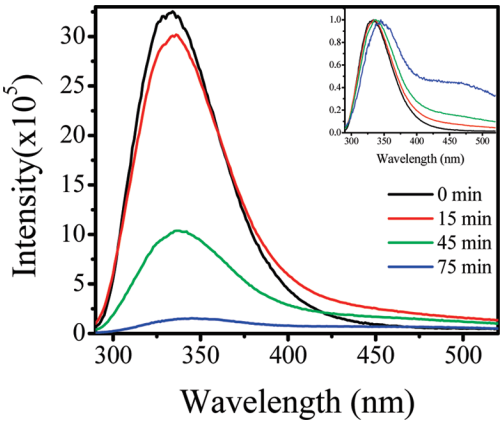

Figure 8. Time-dependent study of steady state fluorescence intensity of cellulase in HEMA in a microwave oven at 65 °C. The samples were excited at 284 nm. The significant quenching and red shift (shown in inset) in the emission suggest that the enzyme is denatured by strong internal heating from the microwave irradiation. Experiments with cellulase in the buffer were not performed, since the enzyme denatures and precipitates at ~50 °C.

reported by Baker et al. On the other hand, the transition temperature of cellulase in HEMA shifted to ~115−125 °C, which suggests that the ionic liquid is increasing its thermal stability. Fused transition peaks were also observed in ionic liquids as in buffer. An additional small transition peak was observed at ~70 °C, which is probably due to early denaturation of a particular component of *T. reesei*. In mim Cl, cellulase showed multiple transition peaks (75 and 94 °C), which are also higher than those observed in pure buffer solution.

Reversibility of the unfolding process was checked by gradual cooling of the denatured enzyme from temperatures greater than the $T_{1/2}$. Cellulase unfolding was irreversible in buffer and accompanied by precipitation of the enzyme. In HEMA, reversibility was observed until 120 °C. The unfolding process was completely irreversible in mim Cl. These observations are consistent with our absorbance studies (Figure 6), confirming that cellulase is more stable and can withstand higher temperatures in the ionic liquids.

Since microwave irradiation has been reported to assist dissolution of cellulose in ionic liquids,[9] cellulase activity in HEMA was also studied using microwave heating at 65 °C, but no liberation of the dye was observed even after 2 h. The fluorescence intensity of cellulase was monitored with time during microwave heating as shown in Figure 8 and was quenched drastically with time, accompanied by a red shift of the peak maxima. This is most likely due to internal heating by microwaves, producing a local temperature at the enzymes greater than 100 °C, resulting in denaturation.

Enzymatic activity is strongly dependent upon the ionic liquid in which it is dissolved. There have been reports that suggest that ionic liquids can deactivate enzymes. Bmim nitrate, bmim lactate, and emim ethylsulfate deactivate enzyme *Candida antarctica* lipase B.[34] Denaturation is likely due to the interaction of different charged groups in the enzyme with the cations and anions in the ionic liquid.[50] Herrmann and co-workers[51] have reported denaturation of Ribonuclease A in different imidazolium-based ionic liquids with different anionic combinations and have argued that the stability of the enzyme in ionic liquids is governed by Hofmeister effects. Anions in ionic liquids have been reported to have a more dominant effect than cations for the stability of enzymes.[52] Those with lower hydrogen bond basicity and nucleophilicity are effective for the stability of the enzymes. Baker et al.[53] studied the protein monellin in bmpyro NTf_2 using steady-state fluorescence and have reported higher thermodynamic stability ($T_m \approx 105$ °C) compared to buffer (~40 °C), which is consistent with our observation with cellulase in HEMA. They have suggested that the ionic liquid might significantly alter the hydration level and compactness of the protein structure. Strikingly, we have found that cellulase was unstable and inactive in bmpyro NTf_2, in which a dramatic quenching of tryptophan fluorescence was observed.

Viscosity plays an important role. As we have seen above, although cellulase is extremely stable and heat resistant in HEMA, its activity is still lower than that in buffer. We suggest that this is because of the high viscosity of the ionic liquid (Table 1). Although cellulase has been reported to be inactive in chloride-containing ionic liquids such as bmim Cl,[36] our studies in mim Cl reveal appreciable activity, which might also be due to its low viscosity relative to other imidazolium ionic liquids with longer alkyl chains, such as bmim Cl. A large viscosity obviously reduces the rate of diffusion, resulting in a lower observed activity of the enzyme. A similar observation by Lozano et al.[54] reveals a reduction in the α-chymotrypsin activity in highly viscous methyltrioctylammonium NTf_2 (574 cP) compared to 1-ethyl-3-methylimidazolium NTf_2 (34 cP). The viscosities of the six ionic liquids (given in Figure 3) in which cellulase did not show any significant activity are all higher than that of mim Cl, but less than that of HEMA. From these data, we suggest that the rate of hydrolysis may depend on the viscosity of the ionic liquid, but it cannot be directly correlated with the activity of the enzyme.

Conclusions

This work provides a detailed study of cellulase activity and stability in various ionic liquids. Among the eight ionic liquids studied here, extensive experiments have been done with mim Cl and HEMA. The former was chosen to compare with the results obtained with bmim Cl reported by Rogers and co-workers.[36] Although cellulase did not fluoresce in mim Cl (consistent with its reported behavior in bmim Cl[36]), it was active in mim Cl, and its activity was higher than that in bmim Cl. The quenching of trytophyl fluorescence in these ionic liquids is due to the high concentration of imidazolium cations and does not necessarily indicate deactivation of the enzyme, as we have observed in the case of mim Cl. The lower viscosity of mim Cl increases enzymatic activity with respect to bmim Cl. Our studies show that in HEMA the enzyme is stable and thermally resistant even at temperatures close to 100 °C, whereas in pH 4.8 buffer, it denatures at ~50 °C. Activity assays and DSC studies prove to be powerful and reliable methods to determine the activity and stability of enzymes in different media. Slight differences in the transition temperatures reported are due to the different techniques used to determine its stability. Although microwave heating can dissolve large quantities of cellulose, the enzyme is denatured under these conditions.

In conclusion, of the eight solvents investigated, HEMA is in many ways the most promising. It is a novel, green medium for performing cellulose hydrolysis reactions to convert biomass into biofuels. Because of the thermal stability it imparts to enzymes, its ability to solubilize biomass, and the fact that it does not quench tryptophyl fluorescence (thus permitting monitoring of the enzymes by fluorescence spectroscopy), HEMA provides an ideal starting point for the design of ionic liquids, not only for the hydrolysis of biomass, but also for use with a wide spectrum of enzymatic reactions. It is also important to investigate the behavior of the *individual* cellulase compo-

nents of *T. reesei*, such as the endoglucanases, cellobiohydrolases, etc. in different ionic liquids. This is part of our ongoing studies.

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