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PAPER

Dependency of the hydrogen bonding capacity of the solvent anion on the thermal stability of feruloyl esterases in ionic liquid systems

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Three feruloyl esterases, EC 3.1.1.73, (FAEs), namely FAE A from *Aspergillus niger* (AnFaeA), FAE C from *Aspergillus nidulans* (AndFaeC), and the FAE activity in a commercial β-glucanase mixture from *Humicola insolens* (Ultraflo L) were tested for their ability to catalyse esterification of sinapic acid with glycerol in four ionic liquid (IL) systems. The IL systems were systematically composed of two selected pairs of cations and anions, respectively: [BMIm][PF_6], [C_2OHMIm][PF_6], [BMIm][BF_4], and [C_2OHMIm][BF_4]. AnFaeA had activity in [PF_6]$^-$-based ILs, whereas the AndFaeC and the FAE in Ultraflo L had no appreciable activities and were generally unstable in the IL systems. FAE stability in the IL systems was apparently highly dependent on enzyme structure, and notably AnFaeA's similarity to IL-compatible lipases may explain its stability. The thermal stability of AnFaeA was higher in buffer than in the IL systems, but at 40 °C and below there was no significant difference in AnFaeA stability between the buffer and the [PF_6]$^-$-based systems: AnFaeA was stable in the [BMIm][PF_6] and [C_2OHMIm][PF_6] systems for 2 h at 40 °C. However, the IL anion had a major effect on stability: [BF_4]$^-$ caused rapid inactivation of AnFaeA, while [PF_6]$^-$ did not. The cation did not have a similar effect. These observations could be explained in terms of the hydrogen bonding capacity of IL cations and anions *via* COSMO-RS simulations.

Introduction

Ferulic acid esterases (FAEs; EC 3.1.1.73) are accessory plant cell wall-degrading enzymes, which catalyse the hydrolysis of the ester bond between ferulic acid and the monosaccharide to which it is covalently linked; in arabinoxylans the ferulic acid is bound *via* *O*-5 bonds to arabinose, whereas linkages of ferulic acid to the *O*-2 of arabinose and *O*-6 of galactose have been shown in pectins.[1] FAEs do however also show activity towards simpler esters (*e.g.* methyl esters) and to a varying degree also towards esters of other hydroxycinnamates such as sinapic acid (SA), caffeic acid (CA), and *p*-coumaric acid (pCA),[2] and the sub-type FAE nomenclature is based on the specific activity on these latter substrates.[3]

FAEs can also be brought to catalyse the (*trans*)esterification reaction in solvents that favour synthesis over hydrolysis, *i.e.* systems with low water content such as organic solvents or ionic liquids (ILs). Recently, modifications of hydroxycinnamates have been performed in order to change their physicochemical and functional properties *via* such enzyme catalysed esterification reactions. These types of reactions may take place either through addition of aliphatic alcohols to increase lipophilicity[4] or by addition of carbohydrates.[5] The reported FAE catalysed esterification reactions are mainly direct esterifications of hydroxycinnamic acids or transesterification reactions of their esters with primary alcohols, *e.g.* 1-butanol, glycerol. However, also reactions with a number of monosaccharides, glycosides, and arabino-oligosaccharides have been demonstrated.[4,5,6] The reactions have been accomplished in microemulsions of organic solvents with low water levels (<5%),[4,5] in nearly solvent-free systems,[6a] or in a single organic solvent.[6b] Hatzakis and Smonou[7] have shown the ability of an FAE from *Humicola insolens* to catalyse the transesterification of vinyl acetate with a number of secondary alcohols in a solvent-free system.

During the last decade, the interest in performing enzyme-catalysed (*trans*)esterification reactions in ionic liquids (ILs) has increased rapidly. This interest has mainly been motivated by a desire to replace volatile organic solvents with non-volatile ILs, which also have the advantage of allowing increased enzyme

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enantio-selectivity and encompass the possibility of solvent tailoring for implementation of new, efficient reaction regimes due to their unconventional solvent properties.[8] To date, most of the enzyme-catalysed reactions in IL systems have been done with lipases, especially lipase B from *Candida antarctica* (CaLB). Stability of the enzymes in the IL matrix is vital when performing these reactions. Although the enzyme stability issue has been addressed in some cases,[9] only Ulbert *et al.*[10] and Lou and Zong[11] have tested the lipase stability (a lipase from *Candida rugosa* and CaLB, respectively) at more than one temperature. Hence, knowledge of enzyme thermal stability in IL systems is scarce. Only a single study has shown the ability of FAE A from *Aspergillus niger* (AnFaeA) to catalyse the (*trans*)esterification of sinapic acid and methyl sinapate with glycerol in an IL-water system using $[C_2OHMIm][PF_6]$ and $[C_5O_2MIm][PF_6]$ with up to 30% of water.[12]

It is an obvious premise for successful catalysis that the enzyme is active in the IL. However, it may be hypothesized that the activity and stability of enzymes will be affected by the reaction temperature and that the stability may vary for different enzyme protein structures. Different ILs may moreover affect the enzyme stability differently. The objective of this work was to elucidate the effect of enzyme structure (and origin) and IL nature on the thermal stability and activity of selected FAEs in IL systems. This was done by determining the synthetic activity and thermal stability of three different FAEs in IL-buffer systems using a carefully selected series of four ILs with pairwise similar anions and cations of varying hydrophobicity and polarity, namely $[C_2OHMIm][PF_6]$, $[C_2OHMIm][BF_4]$, $[BMIm][PF_6]$, and $[BMIm][BF_4]$ (Fig. 1). The choice of $[C_2OHMIm][PF_6]$ was based on the results previously obtained with FAE;[12] the $[BMIm][PF_6]$ and $[BMIm][BF_4]$ ILs were included because they are currently used widely in enzyme catalysis research – although not with FAEs – whereas $[C_2OHMIm][BF_4]$ was included to complete the series. Furthermore, the study aims to explore the use of the quantum chemistry-based COSMO-RS method for explaining and predicting the effect of given ILs on enzyme stability to provide a first foundation for predicting the optimal IL for a particular FAE-catalysed esterification reaction.

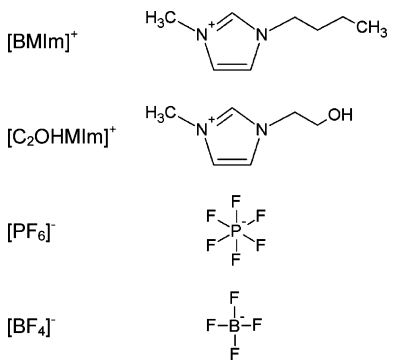

Fig. 1 Structures of the ionic liquid cations and anions used in this study: 1-butyl-3-methylimidazolium ($[BMIm]^+$), 1-(2-hydroxyethyl)-3-methylimidazolium ($[C_2OHMIm]^+$), hexafluorophosphate ($[PF_6]^-$), and tetrafluoroborate ($[BF_4]^-$).

Materials and methods

Materials

Feruloyl esterase type A from *Aspergillus niger* and Ultraflo L (the latter is a commercial β-glucanase mixture from *Humicola insolens*) were provided by Novozymes A/S (Bagsværd, Denmark). Feruloyl esterase type C from *Aspergillus nidulans* (AN5267.2) was produced by fermentation essentially as described by Stratton *et al.*[13] The *Pichia pastoris* clone transformed with the feruloyl esterase gene was obtained from the Fungal Genetics Stock Center as described by Bauer *et al.*[14] Methyl sinapate (methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoate) was purchased from Apin Chemicals (Abingdon, UK). Anhydrous glycerol was purchased from AppliChem (Darmstadt, Germany). LC-MS grade methanol for HPLC analysis was purchased from Fischer Scientific (Loughborough, UK). Diethyl ether was purchased from Merck (Darmstadt, Germany) and $MgSO_4$ from Riedel-de Haën (Seelze, Germany). All other chemicals, including sinapic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) and the ionic liquids $[BMIm][BF_4]$ (1-butyl-3-methyl imidazolium tetrafluoroborate) and $[BMIm][PF_6]$ (1-butyl-3-methyl imidazolium hexafluorophosphate) (purity ≥97%), were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of $[C_2OHMIm][PF_6]$ and $[C_2OHMIm][BF_4]$

Ionic liquids 1-(2-hydroxyethyl)-3-methylimidazolium hexafluorophosphate, $[C_2OHMIm][PF_6]$, and 1-(2-hydroxyethyl)-3-methylimidazolium tetrafluoroborate, $[C_2OHMIm][BF_4]$, were prepared according to the method described by Branco *et al.*[15]

Feruloyl esterase activity assay on methyl sinapate

The activity assay was based on the one proposed by Juge *et al.*[16] In brief, 50 µL enzyme solution was added to 500 µL of a preheated (37 °C) solution of 1 mM methyl sinapate (MSA) in 100 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer (pH 6.0) to start the reaction. After 10 min of reaction at 37 °C, the reaction was stopped by adding 200 µL glacial acetic acid. Amounts of substrate (MSA) and product (sinapic acid; SA) were analysed by RP-HPLC (see below). Activity (U) is expressed as the amount of enzyme required to release 1 µmol of sinapic acid per minute at 37 °C and pH 6.0.

Thermal stability

To mimic the system used for the esterification reaction, the FAEs were incubated in an IL system containing 15% (v/v) enzyme solution diluted in 100 mM MOPS buffer (pH 6.0). Each reaction was conducted in an Eppendorf tube at 700 rpm and at 30 °C, 40 °C, 50 °C, or 60 °C in a Thermomixer (Eppendorf, Hamburg, Germany) for up to 2 h. After 0, 10, 20, 30, 60, and 120 min of incubation, enzyme samples (50 µL; 11.2 mU) were taken out and tested in the feruloyl esterase activity assay at 50 °C. Residual activity was also measured in the esterification reaction in $[C_2OHMIm][PF_6]$ (see below) after 0, 30, and 60 min of incubation in $[C_2OHMIm][PF_6]$ at 40 °C and 50 °C. Control samples were run without enzyme and no reaction was detected.

Enzyme catalysed esterification of glycerol with sinapic acid

Using the results obtained by Vafiadi *et al.*[12] in the esterification of glycerol with sinapic acid using FAE from *A. niger*, the esterification activity of each of the FAEs was tested in a system with 2.5 M glycerol, 0.02 M sinapic acid (solubilised in the IL from its solid form), and 15% (v/v) enzyme solution in 100 mM MOPS buffer (pH 6.0) to give 56 mU. Hence, all reaction mixtures contained 15% (v/v) aqueous buffer, 18% (v/v) glycerol, and 67% (v/v) IL. The total reaction volume was 600 μL, and the reaction took place in an Eppendorf tube kept at 1400 rpm and 40 °C (30 °C when using BF_4-based ILs), in a thermomixer (lower temperature than Vafiadi *et al.*[12] to ensure enzyme stability). The reaction was stopped by extracting substrate and product with ethyl acetate (40 μL sample in 1 mL ethyl acetate) for 4 min at 40 °C and 1400 rpm. After evaporation of the ethyl acetate extract, the remaining solids were re-dissolved in 1 mL of a 1 : 1 methanol-water solution and analysed by RP-HPLC (see below). Control samples were run without enzyme or without glycerol, and no conversion was detected. Conversion yields were calculated from the molar amount of glycerol sinapate formed compared to the molar amount of sinapic acid originally present.

Analysis of reaction components by RP-HPLC

Quantitative analyses were made by RP-HPLC using a Chemstation 1100 series, Hewlett Packard equipped with a C18 column (150 mm × 4.6 mm, 3 μm; Phenomenex (Torrance, CA, USA)) with DAD detection of the substrate and product with quantification at 320 nm. Pure sinapic acid and methyl sinapate were used as external standards. Elution was conducted at 30 °C with [methanol] : [10% (v/v) acetic acid in water] ([4] : [6]) as the mobile phase at a flow rate of 0.5 mL min^{-1} for analysis of the hydrolysis reaction and 0.3 mL min^{-1} for analysis of the esterification reaction. Samples were filtered through a 0.2 μm syringe tip filter prior to analysis.

Generation of ionic liquid σ-profiles by COSMO-RS

The COSMO-file for $[C_2OHMIm]^+$ was made with Gaussian03 (Gaussian Inc. (Wallingford, CT, USA)).[17] The structure was first optimised with the semi-empirical PM3 method and then refined with B3LYP, first with the basis set 6-31 and then with 6-311++G(d,p). After optimisation the two conformers of $[C_2OHMIm]^+$ were almost identical with regards to σ-profiles, and thus only a single one was used here. σ-profiles were generated with COSMO*therm* (COSMO*logic* (Leverkusen, Germany))[18] using the BP-TZVP parameterisation.

Comparison of FAE sequence and structure

Initial sequence similarity searches were performed with protein-protein BLAST using the PSI-BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).[19] Structure similarity searches for homologous proteins were performed by HHPred (http://toolkit.tuebingen.mpg.de/hhpred)[20] using default settings.

Statistics

One-way ANOVA for determination of statistical significance was made in Minitab 16 (Minitab Inc., State College, PA, USA). Statistical significance was established at $p < 0.05$.

Results and discussion

Effect of FAE structure on activity in IL-buffer systems

Among the four ILs used, [BMIm][PF_6], [C_2OHMIm][PF_6], [BMIm][BF_4], and [C_2OHMIm][BF_4] (Fig. 1), the system with [BMIm][PF_6] formed two phases due to the water-immiscibility of this IL. Enzyme activity was found in the aqueous phase of this two-phase system only (data not shown). The other three ILs were water-miscible and only one phase was formed in each of these IL reaction systems.

The AndFaeC was inactivated immediately in the $[C_2OHMIm]^+$-based IL systems, and in less than 10 min in the $[BMIm]^+$-based IL systems. Consequently, only minor esterification activity, *i.e.* ~1% conversion, was seen and only in the [BMIm][PF_6] system (Table 1). However, in MOPS buffer, pH 6.0, the AndFaeC was completely stable for more than 2 h at 40 °C (data not shown). This indicated that the AndFaeC was very sensitive to the IL environment. Similarly, the FAE activity in the Ultraflo L preparation was inactivated immediately in the $[C_2OHMIm]^+$-based IL systems, and in less than 10 min in the [BMIm][BF_4] system (Table 1). In [BMIm][PF_6], however, the FAE activity present in the Ultraflo L was stable throughout the 30 min of reaction (data not shown). This stability was possibly due to the enzyme being present in the aqueous phase in this two-phase system, rather than in the IL matrix. Despite being

Table 1 Conversion (%) of sinapic acid to glycerol sinapate after 30 min of reaction at 40 °C for $[PF_6]^-$-based ILs and 30 °C for $[BF_4]^-$-based ILs in an ionic liquid-buffer (15% v/v) system by 56 mU FAE from three different sources: FAE A from *Aspergillus niger* (AnFaeA), FAE from *Humicola insolens* found as a side activity in the commercial β-glucanase mixture Ultraflo L, and FAE C from *Aspergillus nidulans* (AndFaeC). The number of phases in each IL-buffer system is determined by visual detection. If complete inactivation has taken place during the reaction this is indicated as follows: i0: complete inactivation occurs immediately (less than 30 s); i10: complete inactivation takes place within 10 min

Ionic liquid	No. of phases	AnFaeA	Ultraflo L	AndFaeC
[BMIm][PF_6]	2	$13 \pm 3\%^{a,x}$	$1.0 \pm 0.1\%^{a,y}$	$1.1 \pm 0.1\%^{a,y}$ (i10)
[C_2OHMIm][PF_6]	1	$21 \pm 2\%^{b,x}$	$0\%^{b,y}$ (i0)	$0\%^{b,y}$ (i0)
[BMIm][BF_4]	1	$0.9 \pm 0.1\%^{c,x}$	$0\%^{b,y}$ (i10)	$0\%^{b,y}$ (i10)
[C_2OHMIm][BF_4]	1	$0\%^{c,x}$ (i10)	$0\%^{b,x}$ (i0)	$0\%^{b,x}$ (i0)

Superscript letters a–c indicate significant difference (one-way ANOVA; $p < 0.05$) between reaction outcomes in different ILs for each enzyme, and letters x and y indicate significant difference between enzymes for each IL.

stable, the esterification activity of the FAE activity in Ultraflo L was very low (1% conversion; Table 1).

In contrast, the AnFaeA exhibited significant esterification activity and catalysed 13% conversion in [BMIm][PF_6] and 21% conversion in [C_2OHMIm][PF_6] (Table 1). The former is remarkable as Vafiadi *et al.*[12] reported that no esterification activity was found in [BMIm][PF_6], using the same enzyme and a similar system. As pointed out by Park and Kazlauskas,[21] impurities in the IL and a resulting shift in pH may be the reason for contradictory results. AnFaeA was stable throughout the reaction time in these two $[PF_6]^-$-based IL-buffer systems, but the enzyme was apparently sensitive to the $[BF_4]^-$ systems. Consequently, only low and insignificant activity, 1% conversion, was obtained in [BMIm][BF_4], where the enzyme had only 36% residual activity after 10 min (Fig. 2a). No esterification activity was observed in [C_2OHMIm][BF_4] (Table 1), where the enzyme was completely inactivated within 10 min. This indicated that it is the $[BF_4]^-$ anion rather than the single-phase system or water-miscible IL to which AnFaeA is sensitive. This anion effect is discussed further below.

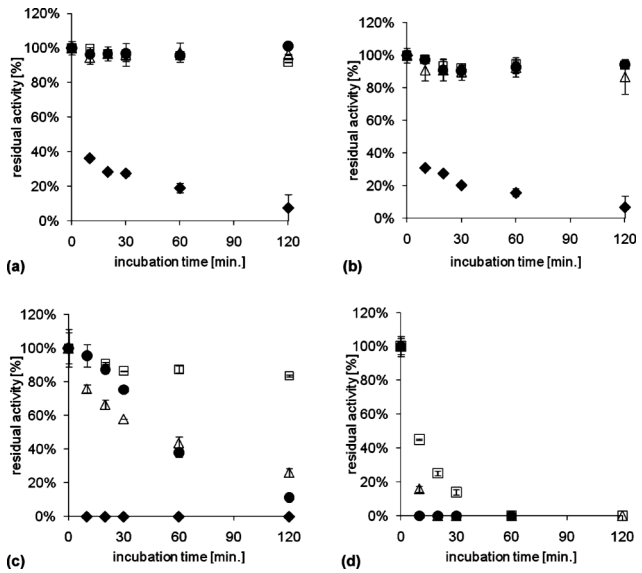

Fig. 2 Thermal stability of FAE A from *A. niger* (AnFaeA) in IL systems: Residual activity of AnFaeA at: (a) 30 °C, (b) 40 °C, (c) 50 °C, and (d) 60 °C when incubated in 100 mM MOPS buffer pH 6.0 (□; *open squares*), [BMIm][PF_6] (●; *filled circles*), [C_2OHMIm][PF_6] (△; *open triangles*), and [BMIm][BF_4] (◆; *filled diamonds*) for up to 120 min as compared to hydrolytic activity at 0 min of incubation. Thermal stability was also tested in [C_2OHMIm][BF_4], but inactivation was immediate (took place in less than 30 s) and for simplicity the data are thus not included in the figure.

The significant difference in esterification activity of AnFaeA observed between [BMIm][PF_6] and [C_2OHMIm][PF_6] is most likely due to differences in water-miscibility and thus water activity as well as viscosity between the two ILs. Generally, water-miscible ILs exhibit a lower water activity, a_w, at a given water content than water-immiscible ILs do.[22] Thus, the supposedly lower a_w of the water-miscible [C_2OHMIm][PF_6] system may in part account for the higher esterification activity. However, at this high water content (15% v/v) even the more hydrophilic $[BF_4]^-$-based systems have an a_w fairly close to 1.[23] Similarly, the a_w of the system containing the more hydrophobic [C_2OHMIm][PF_6] will therefore not be dramatically different from the a_w of the water-immiscible systems which reach maximum a_w = 1 at much lower water contents.[24] Even with 18% (v/v) glycerol in the reaction mixture, the effect of the differential viscosity between the ILs (67% (v/v) of the reaction mixture) cannot be ignored, and the markedly higher viscosity of [BMIm][PF_6] compared to [C_2OHMIm][PF_6] is likely to cause lower reaction rates through mass transfer limitations.[25] The interface formed in the two-phase system with [BMIm][PF_6], the aqueous enzyme solution, and glycerol, may also induce lowered mass transfer and decrease the esterification rate. For an IL-buffer batch system it is therefore advisable to use an IL which is water-miscible and still contains the stabilising $[PF_6]^-$ anion, *e.g.* [C_2OHMIm][PF_6]. From these results it can therefore be concluded that the AnFaeA exhibited potential for being used for enzyme catalysed esterification reactions in an IL-buffer system, whereas AndFaeC and Ultraflo L did not.

All three FAEs have pH optima in the pH 5–6 range (Table 2). AnFaeA and Ultraflo L have higher temperature optima, 55–60 °C and 60–65 °C, respectively, than AndFaeC (37 °C) (Table 2). AndFaeC was also found to be less thermally stable in MOPS buffer (pH 6.0) than the other two FAEs (data not shown). It was thus to be expected that AnFaeA and Ultraflo L would be more stable, at least in the [BMIm][PF_6] system (Table 1).

Remarkably, the feruloyl esterase activity in Ultraflo L had the lowest affinity towards MSA, the substrate towards which AnFaeA and AndFaeC had the highest affinity. Since the FAEs were dosed according to their hydrolytic activity on MSA, this cannot explain the low esterification activity of Ultraflo L in [BMIm][PF_6]. However, if the FAE from *H. insolens* found in Ultraflo L is similar to the one found in Pentopan 500 BG (also a Novozymes blend), which Hatzakis and Smonou[7] found to have affinity for secondary alcohols, this may in part explain why

Table 2 Properties of ferulic acid esterase A from *A. niger* (AnFaeA),[26] ferulic acid esterase from *Humicola insolens* found in Ultraflo L,[27] and ferulic acid esterase C from *A. nidulans* (AndFaeC)[14]

	AnFaeA	Ultraflo L	AndFaeC
Number of amino acids[a]	260	273	249
pH optimum	5	~5–6	6.1
Temperature optimum	55–60 °C	60–65 °C	37 °C
Substrate affinity	MSA > MFA > MpCA	MCA > MFA > MpCA > MSA	MSA[b] > MFA

MSA: methyl sinapate; MFA: methyl ferulate; MpCA: methyl *p*-coumarate; MCA: methyl caffeate.[a] In the final enzyme, without signal peptide. [b] MSA tested in this paper; MCA and MpCA not tested.

the activity is lower in the current system. It cannot be excluded that AndFaeC and the feruloyl esterase activity in Ultraflo L might express higher esterification activity at lower water content than AnFaeA, which has been found to have its optimum at the 15% (v/v) used here. However, since AndFaeC and the feruloyl esterase activity in Ultraflo L also showed low stability in the IL systems (Table 1), it seems more relevant to study the structural differences between the three enzymes.

Most importantly, the three FAEs differ in their number of amino acids (aa.), indicating differences in their overall structure. It has already been established that AnFaeA (260 aa.) has sequence and structure similarities to fungal lipases, especially the open form of the lipases from *Rhizomucor miehei* (37% sequence identity) and *Thermomyces lanuginosis* (30% sequence identity).[28] These particular lipases have previously been found to work fairly well in ionic liquid systems, albeit not as well as the very robust lipase B from *Candida antarctica*.[29] In contrast, AndFaeC (249 aa.) shows a sequence similarity to other (feruloyl) esterases (PSI-BLAST) and when predicting a structure of the enzyme by homology modelling using HHPred, the best match is the ferulic acid esterase domain of the cellulosomal xylanase Z (XynZ) in *Clostridium thermocellum* (sequence identity 21%; E-value 1.6×10^{-26}). Hermoso *et al.*[28] found that XynZ has poor homology in both sequence and structure with AnFaeA. Although AnFaeA and the FAE domain of XynZ differ in overall structure, their Ser-Asp-His catalytic triads in the active site are identical and they both present a long and narrow substrate-accommodating cavity in contrast to the wide and short ones generally found in lipases.[28] Thus, AnFaeA is similar in catalytic mechanism to the FAEs, but similar in structure to some of the fungal lipases – a feature that may explain its higher stability in IL-buffer systems as compared to the other FAEs tested here.

The ferulic acid esterase from *H. insolens* found in Ultraflo L (273 a.a.)[27b] showed sequence similarity to feruloyl and acetyl xylan esterases (PSI–BLAST), and structurally it also has some similarity to the FAE domain of XynZ from *C. thermocellum*, albeit less than AndFaeC (sequence identity 16%; E-value 3.4×10^{-23}). In conclusion, the fact that AnFaeA has a structure more similar to IL-compatible lipases than the other FAEs does seem to be determining for its ability to work well in IL systems.

Effect of IL nature on enzyme thermal stability

Only the FAE A from *A. niger* showed appreciable esterification activity in the IL-buffer systems, and was thus chosen for thermal stability tests at 30–60 °C in [BMIm][PF_6], [C_2OHMIm][PF_6], [BMIm][BF_4], and [C_2OHMIm][BF_4] containing 15% (v/v) buffer.

Thermal stability has been assessed by determining residual activity in an aqueous medium after incubation in ILs. It has been found that CaLB to some extent (33–73%) refolded upon addition of excess water after having been incubated in denaturing ILs.[30] Later, the same group found denaturation of CaLB to be irreversible in another denaturing IL, and concluded that enzyme inactivation in ILs includes a first reversible step and a second irreversible one.[31] The irreversibility of enzyme denaturation in IL systems may (in part) be caused by aggregation of the denatured enzymes.[32] Since any reversibility in the denaturation, *i.e.* re-activation, of AnFaeA, for example upon transfer to an aqueous buffer system for activity measurement, would introduce errors in the thermal stability results, the thermal stability of AnFaeA was also determined as residual esterification activity in the [C_2OHMIm][PF_6] system upon incubation in [C_2OHMIm][PF_6] for up to 1 h (Fig. 3). The data confirmed that there was no significant difference in thermal stability of AnFaeA when measured as residual hydrolytic activity and when measured as residual esterification activity. It can thus be concluded that the temperature-induced inactivation of AnFaeA in [C_2OHMIm][PF_6] is not reversible, and that the thermal stability could be assessed based on hydrolytic activity as well as esterification activity.

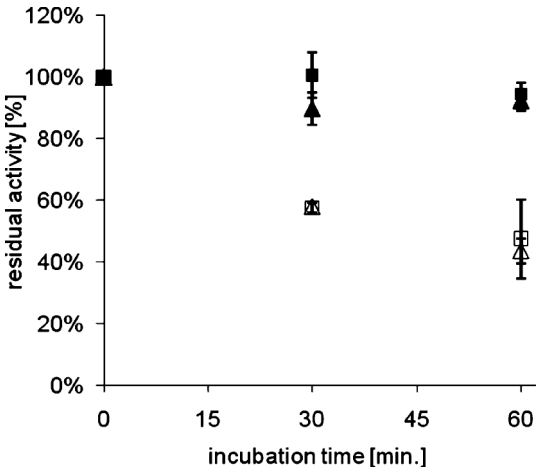

Fig. 3 Residual activity of FAE A from *A. niger* (AnFaeA) after incubation in [C_2OHMIm][PF_6] for up to 1 h at 40 °C measured as hydrolytic activity (▲; *filled triangles*) and as esterification activity in [C_2OHMIm][PF_6] (■; *filled squares*), and at 50 °C measured as hydrolytic activity (△; *open triangles*) and as esterification activity in [C_2OHMIm][PF_6] (□; *open squares*) as compared to the respective activities at 0 min of incubation.

At 30 °C and 40 °C, AnFaeA retained full activity for over 2 h in [BMIm][PF_6] and [C_2OHMIm][PF_6] and there was no significant difference between the stability in the [PF_6]$^-$-based IL systems and stability in the pH 6.0 buffer (Fig. 2a,b). However, at 50 °C AnFaeA was more stable in buffer (k_D = 0.0012) than in [BMIm][PF_6] (k_D = 0.0191) and [C_2OHMIm][PF_6] (k_D = 0.0105). For more than 30 min of incubation, AnFaeA showed significantly higher residual activity in [BMIm][PF_6], but after 2 h the activity was significantly higher in [C_2OHMIm][PF_6]. After 2 h at 50 °C, the residual activity of AnFaeA was 84% in buffer, 26% in [C_2OHMIm][PF_6], and 11% in [BMIm][PF_6] (Fig. 2c). At 60 °C, inactivation was rapid in all media: complete inactivation took place within 10 min in [BMIm][PF_6], within 20 min in [C_2OHMIm][PF_6] (k_D = 0.184), and within 1 h in buffer (k_D = 0.0646) (Fig. 2d).

In contrast, AnFaeA was highly unstable in the [BF_4]$^-$-based IL systems. Even at 30 °C and 40 °C inactivation of AnFaeA was significant in [BMIm][BF_4]: the residual activity was 36% after 10 min and 8% after 2 h at 30 °C (Fig. 2a; k_D = 0.0174), and 31% after 10 min and 7% after 2 h at 40 °C (Fig. 2b; k_D = 0.0179). At 50 °C, complete inactivation took place within 10 min (Fig. 2c). In the [C_2OHMIm][BF_4] system, AnFaeA was

completely inactivated in less than 30 s (data not shown). The results emphasise that just by changing the anion from $[PF_6]^-$ to $[BF_4]^-$ while maintaining the same cations, the effect of the IL on AnFaeA stability changes dramatically. As previously found for lipases,[9a,33] the anion has the dominant effect on FAE stability, and the choice of IL is thus of crucial importance when designing an enzyme-IL system for FAE catalysed esterification reactions. Importantly, the effect of $[BF_4]^-$ anion in particular has been subject to some debate, since some studies have found [BMIm][BF_4] to have a significantly negative effect on the stability of CaLB (Novozym 435),[9b,34] whereas Lau *et al.*[9a] reported full activity and no enzyme dissolution using the same CaLB and [BMIm][BF_4]. Again, different levels of IL purity may explain these contradictory results.[21] For FAEs, however, $[BF_4]^-$ does seem to have a significant, detrimental effect.

In a recent review, Zhao[35] listed a number of IL properties that are likely to influence enzyme stability and activity in IL media, namely polarity, hydrogen bond basicity, nucleophilicity, hydrophobicity, viscosity, and in aqueous IL systems also ion kosmotropicity. Even if no solid prediction tool for enzyme-IL compatibility has been developed due to the complex nature of IL-enzyme systems, there seems to be general consensus that low hydrogen bond basicity and nucleophility as well as high viscosity and especially high hydrophobicity all favour enzyme stability and activity in ILs.[35] Increasing viscosity may however also decrease the reaction rate of the enzyme.[25] Similarly, it has been established that the relationship between enzyme activity and hydrophobicity follows a bell-shaped curve: activity increases with hydrophobicity up to a certain point, but then decreases due to a more thermodynamic ground state stabilization of the substrates and thus less tendency to react.[36] No clear relationship between enzyme activity and polarity has been established, although one study indicated that the reaction rate is higher in more polar ILs.[37] In general, it is believed that the anion has a larger effect on enzyme stability than the cation does,[31] and that large anions with delocalised negative charge on more atoms are generally more stabilising.[38] Larger anions have also been suggested to be less destabilising because they are sterically demanding, in that their size would require many hydrogen bonds in the protein matrix to be broken in order to form a few new ones, making such an interaction less favourable.[9a]

Although hydrophobicity, nucleophilicity, and hydrogen bond basicity are separate properties that should not be confused with each other, the general picture is that the more hydrophilic IL anions are also the ones with the higher nucleophilicity and hydrogen bond basicity, rendering them more likely to interact with positively charged sites in the enzyme thus causing it to change conformation – and *vice versa*.[35] Generally, hydrophobic ILs with $[PF_6]^-$ and $[Tf_2N]^-$ (bis(trifluoromethanesulfonyl)amide) anions are stabilising while hydrophilic ILs with $[NO_3]^-$, [lactate]$^-$, $[TfO]^-$ (trifluoromethanosulfonate), $[EtSO_4]^-$, and in some cases also $[BF_4]^-$ are destabilising to enzymes.[9] The thermal stability results obtained in this work (Fig. 2) support this hypothesis and are the first to be presented for FAEs.

In aqueous systems of hydrophilic ILs such as the $[BF_4]^-$-based systems used here, where the IL is hydrated and dissociates into individual ions, the importance of having a chaotropic cation and especially a strong kosmotropic anion has been pointed out.[39] Therefore, the chaotropic nature of the $[BF_4]^-$ anion may also play an important role in the FAE inactivation in the IL-buffer system. The same tendency has not been observed for hydrophobic ILs which have low solubility in water and therefore limited ion dissociation,[35] explaining why the $[PF_6]^-$ anion does not cause enzyme inactivation despite its highly chaotropic nature.[40]

Cation hydrophobicity has also been found have an effect on enzymes since enzyme enantioselectivity and stability decreases with decreasing alkyl chain length on imidazolium cations from $[OMIm]^+$ to $[BMIm]^+$.[11] This effect may, however, also be confounded with the viscosity effect since a longer alkyl chain length in substituents on the imidazolium cation results in higher viscosity in the range mentioned above (4–8 carbons).[41] This cation effect may be restricted to very similar cations like the ones used in the studies mentioned above. At least, it does not extend to the ones used in this work: [BMIm][PF_6] has higher viscosity and hydrophobicity than [C_2OHMIm][PF_6], but this does not affect the stability of AnFaeA in the two media. There may have been an effect for Ultraflo L (see Table 1), but this may also be explained by the two-phase system formed with [BMIm][PF_6], and this difference in water-miscibility is indeed the major cation effect observed in this work.

Potential of the COSMO-RS method for explaining and predicting FAE stability in ILs

The quantum chemistry-based method COSMO-RS has been introduced as a fast way of performing semi-quantitative tasks such as solvent screening, *e.g.* for substrate solubility, especially for complex media like ILs since it has the advantage over the classical group contribution methods (*e.g.* UNIFAC) that it is not limited to interpolation and partial extrapolation based on available data.[18] Therefore, its ability to explain the effects of the four different ILs used in this study on FAE stability has been tested. The COSMO-RS method calculates the polarisation or screening charge density (SCD), σ, which can be seen as a local measure of polarity for each molecule. The frequencies of screening charge densities ranging from $\sigma = -3$ e nm^{-2} to $\sigma = 3$ e nm^{-2} on the $[BMIm]^+$ and $[C_2OHMIm]^+$ cations and $[PF_6]^-$ and $[BF_4]^-$ anions – also known as σ-profiles – have been plotted in Fig. 4 along with the σ-profile for FAE's 'natural' solvent, water, for comparison. The hydrogen bonding threshold is $\pm\sigma_{HB} = \pm 0.79$ e nm^{-2}, but as hydrogen bonding is weak below ± 1 e nm^{-2} only surface segments with an σ-value beyond ± 1 e nm^{-2} are considered strongly polar and potentially hydrogen bonding.[18] From the σ-profiles it is seen that the peak SCD of $[BF_4]^-$ is found outside the hydrogen bonding limit, whereas the peak SCD of the more hydrophobic $[PF_6]^-$ is inside this limit (Fig. 4). Thus, the destabilising effect of $[BF_4]^-$ on the FAEs reported here can be explained by the tendency of $[BF_4]^-$ to act as a hydrogen bond acceptor and thus interact with the enzyme and disturb its hydrogen bond-based structure. It should also be noted that the SCD peak at $\sigma > 1$ e nm^{-2} is much larger for $[BF_4]^-$ than for water, thus explaining why the IL anion has a destabilising effect while water does not. Although the difference in water-miscibility between the two cations is harder to account for by the σ-profiles (the contribution of the hydroxyl group in $[C_2OHMIm]^+$ can be seen in the range from 0.5 to 1 e nm^{-2}), the

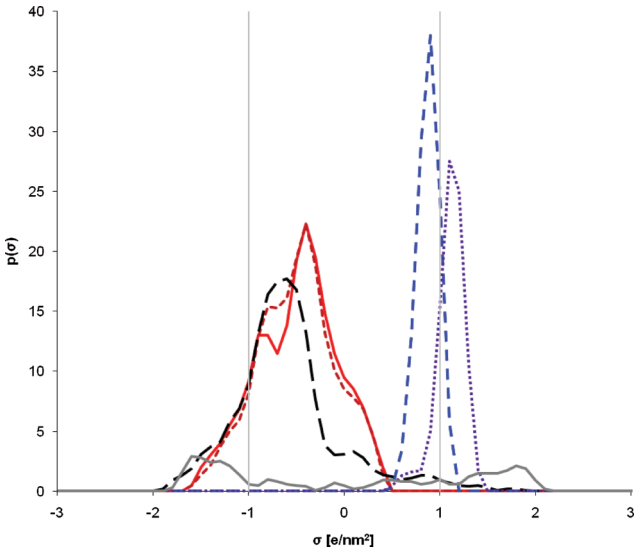

Fig. 4 Sigma(σ)-profiles of the cations and anions in the ILs used: $[BMIm]^+$ (conformer 0 (—; *solid red line*) and conformer 1 (---; *short maroon dashes*)), $[C_2OHMIm]^+$ (— —; *long black dashes*), $[PF_6]^-$ (– – –; *blue dashes*), and $[BF_4]^-$ (· · · ·; *purple dots*). Water (— *solid grey line*) is included for comparison. The vertical lines indicate the hydrogen bonding (HB) limits, *i.e.* screening charge densities outside these lines indicate the tendency to form hydrogen bonds. σ-Profiles are shown for two conformers 0 and 1 of $[BMIm]^+$; COSMO*therm* estimates that in this IL-buffer system (15% (v/v) water), 85.0% of the $[BMIm]^+$ molecules are in the conformer 0 form, while 10.2% are in the conformer 1 form. The two conformers generated for $[C_2OHMIm]^+$ had almost identical σ-profiles; thus, only one is shown.

fact that no major difference in the effect on AnFaeA stability was observed between $[BMIm]^+$ and $[C_2OHMIm]^+$ is consistent with the two cations having similar σ-profiles in the hydrogen bond donor range ($\sigma < -1$ e nm^{-2}).

Other studies have also found COSMO-RS to be a useful tool for solvent screening in IL-based biocatalysis. For example, Lue *et al.*[42] studied enzymatic flavonoid acylation with CaLB in numerous different ILs successfully screened for flavonoid solubility with COSMO-RS and found the method useful for choosing an IL giving the right balance between substrate solubility and enzyme stability.

Conclusions

Of the three FAEs tested here, AnFaeA showed the best potential for being used for esterification in an IL-buffer system, whereas AndFaeC and Ultraflo L were practically inactive in the systems. AnFaeA has a structure more similar to IL-compatible lipases than the other FAEs, and this seems to be determining for its ability to work well in IL systems. The stability of AnFaeA in the IL-buffer system was found to be anion dependent: AnFaeA was rapidly inactivated in the $[BF_4]^-$-based IL systems, but stable in the $[PF_6]^-$-based ones, which were only significantly more destabilising than buffer at 50 °C and above. The $[PF_6]^-$-based IL systems can thus be used with success at temperatures up to 40 °C. Whether the water-miscible $[C_2OHMIm][PF_6]$ or the water-immiscible $[BMIm][PF_6]$ should be used depends on the nature of the desired system – in some cases a two-phase system may be preferable. It may be valuable to assess the effect of simple stabilisation methods such as formation of cross-linked enzyme aggregates (CLEAs) on FAE stability, since this has been found to have a positive effect on CaLB stability in ILs.[31]

The COSMO-RS method proved to be a useful tool for explaining the effect of the four ILs studied in this work on AnFaeA stability in terms of hydrogen bonding capacity. Given the importance of hydrogen bonds in maintaining enzyme structure, the COSMO-RS method may thus prove to enable the prediction of useful ILs for enzymatic reactions in terms of stability. Further tests need to be made on other ILs in order to establish the actual potential, but the four ILs used in this study are good representatives of the ILs commonly used in the field. Although other aspects of IL nature such as water miscibility may not be predicted by COSMO-RS, the method can still be used for initial IL screening to give an indication of substrate solubility and enzyme stability in the IL medium.

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