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Inhibition of hyperthermostable xylanases by superbase ionic liquids

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Short title: Tolerance of thermostable xylanases to ionic liquids

Electronic supplemental material available: Molecular docking figures, graphs for the
calculation of kinetic parameters in ILs and calibration graphs for xylose.

Abstract

The use of enzymes in aqueous solutions of ionic liquids (ILs) could be useful for the enzymatic treatment of lignocellulose. Hydrophilic ILs that dissolve lignocellulose are harmful to enzymes. The toleration limits and enzyme-friendly superbase IL combinations were investigated for the hyperthermophilic *Thermopolyspora flexuosa* GH10 xylanase (4- β -D-xylan xylanohydrolase EC 3.2.1.8) TfXYN10A and *Dictyoglomus thermophilum* GH11 xylanase DtXYN11B. TfXYN10A was more tolerant than DtXYN11B to acetate or propionate-based ILs. However, when the anion of the ILs was bigger (guaiacolate), GH11 xylanase showed higher tolerance to ILs. 1-Ethyl-3-methylimidazolium acetate ([EMIM]OAc), followed by 1,1,3,3-tetramethylguanidine acetate ([TMGH]OAc), were the most enzyme-friendly ILs for TfXYN10A and [TMGH]⁺-based ILs were tolerated best by DtXYN11B. Double-ring cations and a large size anion were associated with the strongest enzyme inhibition. Competitive inhibition appears to be a general factor in the reduction of enzyme activity. However, with guaiacolate ILs, the denaturation of proteins may also contribute to the reduction in enzyme activity. Molecular docking with IL cations and anions indicated that the binding mode and shape of the active site affect competitive inhibition, and the co-binding of cations and anions to separate active site positions caused the strongest enzyme inhibition.

Keywords: biocatalysis; GH10 xylanase; GH11 xylanase; enzyme kinetics; enzyme inhibition; ionic liquid

Introduction

The valorisation of renewable lignocellulosic materials from agriculture and forestry is a global approach to reducing the dependence on fossil resources [1]. The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin [2]. The enzymatic treatment of lignocellulosic carbohydrates generates hexose and pentose sugars [3]. However, the high crystallinity of cellulose and the complex association of polymers in lignocellulose matrix limit the performance of enzymes [4, 5, 6]. Lignocellulose processing also liberates compounds that inhibit enzymes, and in high biomass loading their concentrations becomes high. In addition, a major enzyme inhibition form is end-product inhibition in a high substrate concentration, and this principle affects the industrial high-solids conditions [7, 8, 9]. Ionic liquids (ILs) have shown great promise in the pretreatment of lignocellulosic biomass in reducing recalcitrance to enzymatic hydrolysis [10, 11]. However, ILs may cause a strong enzyme inhibition effect. Thus, studying ways in which to minimize the role of inhibitory factors could be essential to developing efficient processes for enzymatic lignocellulose hydrolysis. ILs are salts that exist in liquid form below 100°C and are useful as dissolving agents in the pretreatment of lignocellulose for improving its enzymatic hydrolysis. Pretreatment with ILs can effectively remove the lignin and reduce the crystallinity of cellulose to permit enzymatic hydrolysis at high solid loadings and low enzyme concentrations [12, 13, 14]. Ionic liquids can also enhance chemical catalysis of cellulose for products [15] and, together with deep eutectic solvents, are favoured as potential green solvents [16, 17]. Hydrophobic ILs are known to stabilize enzymes, whereas those ILs of a hydrophilic nature are highly detrimental to enzymes [18, 19]. The incompatibility of enzymes with ILs enhances the need for extensive washing of the cellulose fibre before the enzymatic hydrolysis [20]. Thus, developing new enzymes with a higher tolerance to

hydrophilic ILs or finding ILs that are more enzyme-friendly is necessary in order to increase the use of hydrophilic ILs together with enzymes.

Xylan is the major constituent of hemicelluloses and the second most abundant polymer after cellulose. Endoxylanases (EC 3.2.1.8.) are glycoside hydrolase enzymes that hydrolyse the cleavage of 1,4- β -D-xylosidic linkages in xylan, leading to the liberation of xylooligosaccharides and xylose. Most of the studied endoxylanases belong to the GH10 and GH11 families. Endoxylanases of the GH10 family have a high molecular weight (>30 kDa) and low isoelectric points (pI), whereas GH11 endoxylanases have a low molecular weight (<30 kDa) and a high pI [21]. Xylanases are used in feed production, pulp bleaching and the food industry [21, 22]. They can also be used to assist cellulases in lignocellulose hydrolysis [23, 24].

ILs affect both enzyme activity and stability [25, 26]. In dilute aqueous solutions, hydrophilic ILs become at least partially dissociated and the solvated ion consequently interact individually with the enzyme [25]. Different enzyme inhibition mechanisms were observed in ILs solutions: competitive [27, 28], uncompetitive [29], pure non-competitive [30, 31] and mixed inhibition [31]. ILs destabilize enzymes via disruption of the protein secondary structure [32, 33]. In dilute IL solutions the kosmotropic effect (Hofmeister series) of the IL ions on enzymes may be applicable: kosmotropic anions and chaotropic cations favour enzyme functioning, while chaotropic anions and kosmotropic cations do not favour it [25]. It is suggested that the specific ion effect is used instead of the Hofmeister series effect because of the debate on whether the hydration of ions perturbs the water structure surrounding the enzymes [25]. Hydrophobicity of ions and polarizability of anions are among the factors that are implicated in the ion-specific effect on enzyme activity and stability in aqueous solutions of ILs [25, 26, 34, 35]. The functioning of certain enzymes in ILs does not

follow the Hofmeister series, which reflects the fact that the kosmotropicity/chaotropicity effect is not the only factor governing enzyme performance [25, 26].

Lignocellulose-degrading enzymes from extremophile prokaryotes exhibit extreme tolerance towards heat, acids, alkali and salts [36, 37, 38]. The use of thermophilic enzymes was among the strategies adopted to promote enzyme tolerance to ILs that dissolve cellulose [36, 37, 39]. Many endoxylanases from hyperthermophilic microorganisms show tolerance towards ILs [27, 39, 40, 41]. In general, GH10 xylanases tolerate more 1-ethyl-3-methylimidazolium acetate ([EMIM]OAc) and other ILs than GH11 xylanases [39, 40, 41, 42]. However, resistance to protein unfolding in thermostable enzymes is not the only factor in IL tolerance [28, 39, 40]. Competitive inhibition was found to be a key factor in xylanases inhibition [27, 28, 40]. The stronger binding affinity of the substrate to the enzyme appears to lower competitive inhibition [27]. The inactivation of a thermostable endoglucanase from *Acidothermus cellulolyticus* in ILs proceeds at 65°C in a biphasic manner. The inactivation begins with a rapid reversible competitive inhibition at all IL concentrations, followed by a slow irreversible protein denaturation after a prolonged incubation time at higher IL concentrations [43]. These phenomena were also observed in xylanases [28, 44].

The objective of the current work was to study the effect of diluted aqueous solutions of various untested ILs on the functioning of highly thermostable endoxylanases belonging to the GH10 and GH11 family. The ILs used were a set of superbase-derived ILs: 1,5-diazabicyclo[4.3.0]non-5-enium acetate, [DBNH]OAc; 1,5-diazabicyclo[4.3.0]non-5-enium propionate, [DBNH]CO₂Et; 1,8-diazabicyclo[5.4.0]undec-7-enium acetate, [DBUH]OAc; 1,8-diazabicyclo[5.4.0]undec-7-enium propionate, [DBUH]CO₂Et; 1-ethyl-3-methylimidazolium acetate, [EMIM]OAc; methyl-1,5-diazabicyclo[4.3.0]non-5-enium dimethyl phosphate, [mDBN]Me₂PO₄; 1,1,3,3-tetramethylguanidinium acetate, [TMGH]OAc; 1,1,3,3-

tetramethylguanidinium propionate, [TMGH]CO₂Et; 1,5-diazabicyclo[4.3.0]non-5-enium 2-hydroxy-3-methoxybenzoate, [DBNH]guaiacolate; 1,8-diazabicyclo[5.4.0]undec-7-enium 2-hydroxy-3-methoxybenzoate, [DBUH]guaiacolate; 1,1,3,3-tetramethylguanidinium 2-hydroxy-3-methoxybenzoate, [TMGH]guaiacolate. The motivation behind the use of these ILs is their cellulose dissolution capability and the recyclability of some of them [45, 46, 47]. Furthermore, by using highly thermostable enzymes, the unfolding effect of ILs on enzymes is unlikely or minimal. Consequently, the effect of ILs on enzyme activity could be studied.

Materials and Methods

Sources of enzymes and ionic liquids

The xylanases studied were *T. flexuosa* XYN10A xylanase TfXYN10A (family GH10) and *D. thermophilum* XYNB xylanase DtXYN11B (family GH11) and its stabilized variant DtXYN11B-DS. The DtXYN11B-DS mutant has an N-terminal disulphide bridge designed between the Cys1 and Cys27 positions [28]. All the enzymes were expressed extracellularly from *Escherichia coli* as previously described [28, 48]. TfXYN10A was 80% pure in SDS-PAGE [48]. The purification of DtXYN11B and DtXYN11B-DS was performed as previously described [28] and the proteins were 70–80% pure in SDS-PAGE (assessed by densitometry) [28]. Ionic liquids based on [DBNH]⁺, [DBUH]⁺, [mDBN]⁺ and [TMGH]⁺ cations were prepared as previously reported [45, 46]. [EMIM]OAc was purchased from BASF (Ludwigshafen, Germany, purity ≥95%). To prepare guaiaculates, 1 eq. of superbase was added to 1 eq. of guaiacol in a round-bottom flask at room temperature, while stirring. The sample was stored under argon and away from light.

Enzyme assays

Xylanase activity was measured by incubating the enzymes with beechwood xylan for 30 mins at 70°C. This temperature was chosen because the ILs reduce the apparent

temperature optimum for activity (temperature optimum for the enzymes was 80°C, 90°C and 95°C for TfXYN10A [27], DtXYN11B and DtXYN11B-DS [28], respectively). The reaction mixture (2 mL) contained 0.2 mL of an appropriate dilution of enzyme in 1.8 mL of 1% (w/v) substrate dissolved in 50 mM citrate-phosphate buffer at optimal pH of enzyme activity; pH 6 for TfXYN10A and pH 6.5 for DtXYN11B and DtXYN11B-DS. 0.1 mg/mL BSA was used as a stabilizer to prevent non-specific enzyme binding to the tube wall. The reaction was stopped by the addition of 3 mL 3,5-dinitrosalicylic acid (DNS), boiled for 5 mins and then the reaction product was measured as previously described [49, 50]. Enzyme activities in the presence of ILs were carried out with 5%, 15%, 25% and 35% IL solutions. The guaiacolate-based ILs formed a brown precipitate at high concentration with the xylan solution. For this reason, these ILs were used at 5% concentrations. Each enzyme was appropriately diluted to generate an absorbance of around 1 at 540 nm in the DNS assay without IL. The specific activity of each enzyme used in the final assay was 803 U/mg for TfXYN10A, 2175 U/mg for DtXYN11B and 848 U/mg for DtXYN11B-DS. In the reactions with 4% substrate or 35% IL, the activity was measured in a 1.0 mL reaction mixture. The reaction was stopped by the addition of cold buffer and the reducing sugars were measured using the DNS method. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μ mol of product per min. ILs that were not liquid at room temperature were melted in a heated water bath before addition of the substrate solution. The addition of ILs to the substrate solutions induced a change in the pH, which was corrected by the addition of HCl or NaOH before the addition of enzyme. The presence of IL in the reaction mixture led to an increase in the absorbance. Consequently, calibration graphs in the presence of ILs were constructed and used to correct the obtained absorbance values (Fig. S1 in the Supplementary Material).

Kinetic experiments

Kinetic parameters were performed for TfXYN10A in standard assay conditions with and without 5% (v/v) IL, using 1, 2, 4, 6, 8, 10, 15 and 20 mg/mL beechwood xylan as substrate (Figs. S2 to S13). Activity was measured at 70°C, pH 6. All the experiments were performed at least three times with triplicates. The kinetic values (unweighted) were calculated by hyperbolic regression analysis function by using the Hyper 32 programme.

Molecular docking

IL cations and anions were docked one by one by SwissDock [51] to the active site of the DtXYN11B structure (PDB code 1F5J [52]) and the modelled structures of TfXYN10A based on the PDB structure 1v6w [48]. The protein and ligand structures included hydrogens (added in Swiss-PdbViewer to proteins). Cation and anion structures were energy minimized by using MM2 in ChemBio3D Ultra 12.0 (CambridgeSoft) or the geometry was optimized by using Avogadro (<http://avogadro.cc/>) (in the case of guaiacolate anion) before the docking experiments, although prior ligand minimization is not required in SwissDock [53]. SwissDock performs the final minimization during the docking [51]. Accurate mode was used in SwissDock and flexibility was permitted for the ligand but not for the side chains. Charges are from MMFF. The SwissDock computed CHARMM binding energies. The binding modes were ranked based on favorable energies, including the solvent effect using the Fast Analytical Continuum Treatment of Solvation (FACTS) implicit solvation model [51, 54]. The obtained results were analysed by using UCSF Chimera [55].

Results

Activity of xylanases in ionic liquids

The activity of a family GH10 xylanase (TfXYN10A) and a family GH11 xylanase and its stabilized variant (DtXYN11B and DtXYN11B-DS, respectively) was studied in a new set of hydrophilic ILs and compared to our previous studies [27, 28, 39, 40]. The two

enzyme families were first compared by measuring their activities in 15% (v/v) concentrations of acetate-propionate ILs and [mDBN]Me₂PO₄ (Fig.1) and in 5% (v/v) concentrations of guaiacolate-based ILs (Fig. 2). TfXYN10A showed high tolerance to most of the studied ILs in 15% concentrations (Fig.1). In contrast, almost all the acetate-propionate ILs and [mDBN]Me₂PO₄ had a drastic effect on the GH11 xylanase DtXYN11B and its stabilized variant (DtXYN11B-DS) (Fig.1). Similar tolerance values were obtained for both enzymes (DtXYN11B and DtXYN11B-DS), indicating that the additional stability of DtXYN11B-DS did not offer any additional benefits to the enzymes in this high thermostability range of the enzymes. Remarkably, [EMIM]OAc, which was tolerated best by GH10 xylanase in 25-35% IL concentrations (Fig. 5). However, [EMIM]OAc was not best tolerated by GH11 xylanases at milder 15% IL concentrations, but the difference was not big.

DtXYN11B and DtXYN11B-DS tolerated the [TMGH]⁺ cation-based ILs best (Figs. 1 and 2). They tolerated [TMGH]guaiacolate even better than TfXYN10A (Fig. 2). We also found that the stabilised variant DtXYN11B-DS was more tolerant to the guaiacolate ILs than DtXYN11B (Fig. 2). Thus, the additional thermostability may account for the higher IL tolerance of DtXYN11B-DS.

Activity of TfXYN10A in 4% substrate

Since many ILs are known to cause competitive inhibition, a higher activity should be achieved in the presence of ILs when using a higher substrate concentration. The substrate would in higher concentration replace better the IL away from the active site. Thus, the activities of TfXYN10A were compared in 1% and 4% substrate concentrations (Figs. 3 and 4). The results showed that the enzyme has much higher activity in 4% substrate than in 1% substrate with all ILs. With acetate-propionate ILs or [mDBN]Me₂PO₄, elevated activity of the enzyme was often achieved in 4% substrate in the presence of ILs when compared to

activity without IL (Fig. 3), indicating that ILs improved the enzyme activity in 4% substrate when the inhibition effect was overcome. However, with guaiacolate ILs, the recovery of enzyme activity by 4% substrate concentration was much lower than the activity without ILs (Fig. 4), indicating that the enzyme suffers inhibition by guaiacolates even at elevated concentration of the substrate. The increase in activity upon moving from 1 to 4% substrate appears to be higher in guaiacolate ILs in most cases (Fig. 4) than in the other ILs (Fig. 3). The stronger inhibition may occur because of the presence of ring structure (benzoic ring) in the guaiacolate anion (Fig. S14), which together with the cation, probably bind better to the active site than the acetate and propionate-based ILs. Another reason could be a stronger denaturing effect of the guaiacolate anion on the enzyme.

Effect of 5–35% ILs on TfXYN10A

The activity of TfXYN10A was further studied in different concentrations of the acetate-propionate ILs and [mDBN]Me₂PO₄ (Fig. 5). The 5% (v/v) IL concentrations of all ILs were well tolerated and 5% [DBNH]CO₂Et and 5% [mDBN]Me₂PO₄ even increased TfXYN10A activity slightly (Fig. 5). A slight enhancement of xylanase activity has been previously observed in xylanase E2 in [EMIM]Me₂PO₄ at all the concentrations used (5, 10, 15, 20% v/v) [56]. [EMIM]OAc, which has been previously studied [27], retained the enzyme activity much better than all the other ILs in 25–35% concentrations. [TMGH]OAc was the second-best tolerated IL. [EMIM]OAc achieved the highest (60%) activity in 35% IL. All other ILs fully inactivated TfXYN10A in 35% concentrations, except 35% [TMGH]OAc, which allowed around 20% activity.

By using a series of ILs based on a set of cations and anions, it was possible to compare the role of each ion in the inhibition. In 25% ILs, the order of decreasing activity follows an order of cations which is the same for both shared anions: [EMIM] > [TMGH] >

[DBUH] > [DBNH]. The order [EMIM]OAc > [TMGH]OAc is preserved at 35% concentrations. However, in 15% ILs, this order was only observed in propionate-based ILs, and not at 5% ILs concentrations (Fig. 5). These findings suggest that a sufficient concentration of ILs is necessary to show a clear order of inhibition.

At 25% concentrations the ILs with propionate anions showed a more drastic negative effect on enzyme activity than ILs with acetate anions. No difference in the effect on enzyme activity was observed between these two anions at 5% or 15% concentrations (Fig. 5). Guaiacolate anion generated the strongest inhibition when studied with [DBNH]⁺, [DBUH]⁺ and [TMGH]⁺ cations at 5% concentrations, while with acetate and propionate these cations were well tolerated by TfXYN10A enzyme at this concentration (Fig. 5). With guaiacolates an inhibition of more than 60% was observed. (Fig. 2).

Effect of ionic liquids on enzyme kinetic parameters of TfXYN10A

Michaelis Menten's steady-state model describes the kinetic reaction of an enzyme with one substrate binding site. In this model, K_M is the concentration of substrate corresponding to half of V_{max} and reflects the enzyme's affinity for the substrate, while V_{max} is the velocity of the reaction at excess substrate concentrations. Thus, the effect of an IL on K_M and V_{max} reflects its effect on binding of the substrate to the active site and its effect on the catalytic rate, respectively.

To get a deeper insight into the effect of ILs on TfXYN10A, the ILs were used at 5% concentrations to study their effect on K_M and V_{max} (Table 1). V_{max} was measured as a relative value, in relation to the value in the absence of IL, which gives a comparative result for the effect of IL. All ILs increased the K_M , but the increase was most pronounced with guaiacolate-based ILs, with [TMGH]guaiacolate resulting in the greatest increase. The V_{max} remained close to similar levels than without the IL for most acetate propionate ILs, except

[DBNH]CO₂Et. [DBNH]CO₂Et and [mDBN]Me₂PO₄ increased the $V_{\max}$, which may explain the slight increase in activity observed at 5% IL concentrations (Fig. 5). The common feature of these two ILs is the DBN core of the cation. [DBNH]CO₂Et showed the highest increase in $V_{\max}$, but despite this, the highest inhibition at higher IL concentrations (Fig. 5). Thus, the concentration-dependent behaviour may differ between different ILs.

The very low increase in K_M with most ILs indicates that TfXYN10A has a very high ability to resist the competition of 5% ILs. The acetate-propionate pairs and [mDBNH]MePO₄ in the low IL concentration that was used did not show any consistent differences in their effect on the kinetic parameters (Table 1). However, the replacement of acetate or propionate anions with the guaiacolate anion leads to a significant difference in the kinetic parameters, especially in the K_M . The guaiacolate-based ILs also clearly decreased the $V_{\max}$ which, in combination with an increased K_M , may explain the low activities observed in these ILs.

Binding of ionic liquid molecules to the active site

SwissDock, which recognizes charge interactions, was used in the molecular docking of the structures of IL cations and anions onto the xylanase structures (IL cations and anions are shown in Fig. S14). The molecular docking of [DBNH]⁺, [DBUH]⁺, [EMIM]⁺, [mDBN]⁺ and [TMGH]⁺ cations and acetate, propionate and guaiacolate anions to TfXYN10A structure, and cations and guaiacolate anion to DtXYN11B structure was performed in order to obtain preliminary information on how IL molecules could bind to the active site and how the observed binding behaviour among the detected 250-256 poses grouped into binding clusters could correlate to the enzyme activity in IL solutions. In TfXYN10A, 4-13 cation clusters (out of 35-50 total binding clusters) and in DtXYN11B 20-33 cation clusters (out of 34-39 total binding clusters) were binding to the active site (Table S1). Each binding cluster represents an overlapping binding of typically several binding poses, but also many clusters showed highly

overlapping binding. All poses of one guaiacolate cluster are placed as an example in Fig. S17A. Number of bound clusters in the active site representing the amount of alternative binding sites quite largely seemed to correlate to the activity inhibition (Table S1). The higher amount of binding cation clusters in DtXYN11B also correlated to the stronger inhibition of DtXYN11B, as seen earlier with [EMIM]⁺ cation [40].

The active site shape is different in TfXYN10A and DtXYN11B, and the extent of IL bindings is different, since the active site of GH11 enzyme is deeper (Figs S15 and S16). As was observed earlier in the docking of [EMIM]⁺ cation to the active site of TasXyn10A and DtXYN11B xylanases [40], the potential IL cation binding areas are much smaller in TfXYN10A than in DtXYN11B (Figs. S15 and S16). This was seen in this study with all IL cations, reflecting the much higher average inhibition of DtXYN11B by different ILs. Basically, only a few cations (around one to four) are likely to fit simultaneously to the active sites based on the available space and the size of cations (Figs. 6 and 7).

In the simulation by Jaeger and Pfaendtner (2013) [44], two or three [EMIM]⁺ cations preferentially occupy space very near to the substrate binding site of GH11 xylanase from *Trichoderma longibrachiatum* [44]. Typically, with all cations, the highest binding energy poses of cations in the active sites were located above the catalytic residues, indicating the higher binding capacity of the corresponding substrate binding site. For example, in the highest binding energy pose of [DBNH]⁺ cation on TfXYN10A (upper cation in Fig. 6), the nitrogen atom of [DBNH]⁺ ring is packed against the catalytic residue Glu128 and the hydrophobic parts of the cation rings pack against nearby aromatic rings of Trp85, Tyr172 and Trp274 on the active site canyon surfaces (not shown).

Jaeger and Pfaendtner (2013) [44] observed a specific interaction in the GH11 xylanase between the positively charged lysine and arginine surface residues and the

negatively charged oxygen atom of acetate and ethyl sulphate anions of the ILs [44]. The docking of anions to GH10 xylanase TfXYN10A revealed that the major anion binding site was at the other end of the active site canyon from where the cations were binding (Figs. 6 and S18). 15 clusters of guaiacolate poses bound into the active site out of total 39 clusters (Table S1), but only two clusters of propionate or acetate. In the anion binding site, the guaiacolate anion is packed against positively charged Arg275 (Fig. 6 and S18A). Acetate and propionate poses were also located close to Arg275 (see Fig. S18B for propionate). In TfXYN10A, based on the size of the active site and sizes of the IL molecules and positions of the potential cation and anion binding sites, the active site space is estimated to be able to simultaneously harbour, for example, two [DBNH]⁺ cations and one guaiacolate anion, which then together are likely to fill the active site. [DBNH]⁺ cation did not bind to the area that binds guaiacolate (Fig. S18C), probably giving space for guaiacolate anion binding.

Docked molecules of guaiacolate and two cations are shown in Fig. 7 for DtXYN11B so that the highest energy binder was placed first and then the other molecules that fit the active site without overlapping were chosen to be shown. The space-filling structures of guaiacolate anions, and [DBNH]⁺ and [TMGH]⁺ cations binding to the active site of DtXYN11B from different clusters are shown in Fig. 7 and the corresponding active site clusters are shown in Fig. S17. In DtXYN11B, the active site contained many poses for guaiacolate (5 clusters), but active site hits for propionate and acetate were only few in distant positions (not shown). The docking results indicated that unlike in TfXYN10A, the anion and cation binding sites appear to be overlapping in DtXYN11B (Fig. 7).

Binding energies of cations did not produce any clear general correlation to activity effect (Table S1). The energy level of the highest energy binders in the active site with guaiacolates compared to acetate/propionate reflected the strength of the inhibition level. The

strongest inhibition was with guaiacolate anions (highest binding energy -7.65 kcal/mol for TfXYN10A and -6.20 kcal/mol for DtXYN11B) and the weakest inhibition was with propionate (-6.50 kcal/mol) and acetate (-6.48 kcal/mol) (propionate and acetate ILs tested only for TfXYN10A). The cation-binding energies were all in the range of -6.4 – -7.0 kcal/mol for both enzymes, indicating that the guaiacolate binding to TfXYN10A could be strongest among these IL molecules.

Discussion

The processing of lignocellulose in high-solids conditions by enzymes for various purposes generates mixtures that contain various molecules that inhibit enzymes. For example, substrate inhibition, product inhibition, binding of enzymes to lignin and inhibition by enzymatic and chemical degradation products, such as oligosaccharides and furfurals, can reduce enzyme efficiency [7, 8, 9]. Thus, enzymes tolerant to inhibiting compounds are important in industrial lignocellulose processing [57, 58]. When ionic liquids (ILs) are used to assist lignocellulose pretreatment, they can inhibit enzymatic hydrolysis in the same way as other inhibitors derived from the biomass. We have studied the inhibition mechanism of ILs on glycosyl hydrolases and found that competitive inhibition appears to be the main reason for reduced enzyme activity [27, 28, 40, 42]. The molecular understanding of competitive inhibition is relevant to the development of better enzymes to be used in biorefineries. Knowledge of the factors that affect enzymes in ILs is of great importance in selecting enzyme-ionic liquid combinations for biorefinery applications.

Xylanases have been studied for their ability to assist cellulases in lignocellulose hydrolysis [23, 24]. Their activity has been tested in hydrophilic ILs that are capable of dissolving lignocellulose. The first findings with [EMIM]OAc indicated that family GH10 xylanases are more tolerant to ILs than GH11 xylanases [27, 28, 40, 41, 42,]. However,

further studies with different xylanases and different ILs are necessary in order to establish this hypothesis as a general principle. Thus, in this study, the kinetic behaviour of a GH10 xylanase and a GH11 xylanase with its stabilized variant were studied in new aqueous IL solutions. The goal was to gain a wider picture of the competitive inhibition caused by ILs, and how these two enzyme families differ in this respect. TfXYN10A was already shown to be the most IL-tolerant enzyme among other GH10 and GH11 xylanases (including DtXYN11B), retaining 100% of relative activity at 60°C in the presence of a 15% concentration of seven hydrophilic ILs ([DMIM]DMP, [BMIM]DBP, [Chol]AcO, [BMIM]DMP, [TMGH]n-PrCOO and [EMIM]DMP) [39]. DtXYN11B retained 49% average activity in this set of ILs, [Chol]OAc being the most tolerated (90% activity) [39]. In the present study, we tested the enzymes at 70°C with seven acetate propionate-based ILs ([DBNH]OAc, [DBNH]CO₂Et, [DBUH]OAc, [DBUH]CO₂Et, [EMIM]OAc, [TMGH]OAc and [TMGH]CO₂Et), three guaiacolate-based ILs ([DBNH]guaiacolate, [DBUH]guaiacolate, [TMGH]guaiacolate) and [mDBN]Me₂PO₄. The results showed that, in general, GH10 xylanase TfXYN10A tolerates this set of ILs better than the GH11 xylanases DtXYN11B and DtXYN11B-DS. [EMIM]OAc, followed by [TMGH]OAc, were the best tolerated by TfXYN10A. [TMGH]OAc followed by [TMGH]CO₂Et and [EMIM]OAc were the best tolerated by DtXYN11B. [TMGH]OAc is a potentially distillable IL [44] and the studied enzymes were very active in it, making it a promising IL for practical application.

Both GH11 xylanases DtXYN11B and DtXYN11B-DS and GH10 xylanase TfXYN10A are highly thermostable and active well above the 70°C used as the assay temperature in this study [27, 28]. Thus, they are not likely to be denatured by low concentrations of ILs at 70°C, since in 35% [EMIM]OAc, TfXYN10A had a temperature optimum of 70°C [27] and in 20% [EMIM]OAc, the temperature optimum of DtXYN11B was

75–80°C [28]. Because of this, the recovery of activity by increasing the concentration of the substrate suggests that competitive inhibition is the main factor in the inhibition of enzymes by the acetate-propionate ILs and [mDBNH]MePO₄ at 15% concentrations. Previous works have studied the activity and thermal stability of the same xylanases [27, 28] or other thermostable xylanases [40, 41] in the presence of a dilute aqueous solution of [EMIM]OAc, [EMIM]DMP and [DBNH]OAc (5–35%). The enzymes appear not to lose as much enzyme stability as activity, and competitive inhibition by the IL ions plays a key role in the loss of activity.

Guaiacol is a common phenolic residue produced in lignin degradation during biomass processing [59] and was shown to exert an inhibitory effect on *Aspergillus japonicus* xylanase [60] and *Trichoderma reesei* cellulase [61]. At 5% concentrations of guaiacolate-based ILs, the finding that the stabilized variant DtXYN11B-DS shows a higher tolerance than the wild type DtXYN11B and that there is no full recovery of activity at high substrate concentration, suggests that the N-terminal disulphide that increases enzyme stability protects against the effect of guaiacolate ILs. Thus, in contrast with other ILs, guaiacolate-based ILs may cause unfolding of the enzyme at the studied temperature. Based on a steady-state (tryptophan) fluorescence spectroscopy study, it was suggested that the inhibitory effect of phenolic compounds, like guaiacolate ILs, on thermostable GH11 xylanase was attributable to structural alterations in the protein [62]. It is therefore possible that protein denaturation was delayed by the stabilizing disulphide bridge in the N-terminus of DtXYN11B-DS, a region from which the unfolding starts in GH11 xylanases [63]. However, further experiments are needed to confirm this hypothesis of protein denaturation.

Two ILs appear to have the lowest inhibition effect on DtXYN11B: [TMGH]OAc in our study and choline acetate [Chol]OAc in the study of Rahikainen et al. (2017) [39].

However, [TMGH]⁺ cation with a large anion molecule showed much stronger inhibition, particularly [TMGH]guaiacolate in our study and [TMGH]n-PrCOO (butyrate) in the study of Rahikainen et al. (2017) [39]. All these results indicate that the effect of the cation is tuned by the properties of anion.

Several properties of IL ions have been found to play a key role in the inhibition of enzyme in dilute solutions of ILs. They include hydrophobicity, polarizability and the size of the ions [25, 26, 34, 35]. The order of increasing inhibition of TfXYN10 by the cations reveals that cations with one ring structure or without ring ([TMGH]⁺ and [EMIM]⁺) are the most tolerated by TfXYN10A, whereas the cations with double-ring structures ([DBNH]⁺, [mDBN]⁺ and [DBUH]⁺) are the most inhibiting (Fig. S14). The most tolerated IL cations contain the smallest amount of hydrophobic C and H atoms ([EMIM]⁺ 17; [TMGH]⁺ 17) and the less tolerated IL cations have a higher amount ([DBNH]⁺ 19; [DBUH]⁺ 25 and [mDBN]⁺ 23). Several studies have indicated that a larger hydrophobic surface in the cation causes stronger enzyme inhibition [39, 35, 34] However, the hydrophobicity of the cations does not fully correspond to the order of inhibition for TfXYN10A xylanase that was observed in our study, suggesting that further factors are also implicated in the inhibition.

The cations used are a set of superbase conjugate acids with varying basicities of the unconjugated superbases: [mDBN]⁺ > [EMIM]⁺ > [DBUH]⁺ > [DBNH]⁺ > [TMGH]⁺ (most to least basic) [45]. [DBNH]⁺ and [DBUH]⁺ were the most acidic cations and were not the most enzyme-friendly, while [mDBN]⁺ was the most basic cation and was not the most tolerated. Thus, properties other than cation acidity-basicity appear to dominate the activity inhibition. There is no correlation in the inhibition of enzymes with the total surface area and cavity volume values (not shown) formed from both polar and nonpolar atoms. Instead, the [EMIM]⁺ cation shows a higher surface area/volume (SA/V) ratio than the others, which could relate to

its milder inhibition level. The SA/V values are: [DBNH]⁺ 1.01; [DBUH]⁺ 0.96; [EMIM]⁺ 1.11; [mDBN]⁺ 1.00 and [TMGH]⁺ 1.02 [45].

The decrease in enzyme activity in the presence of propionate compared to acetate anion at 25% concentrations may be attributed to the increasing alkyl chain length of the anion (acetate compared to propionate), which corresponds to an increase in hydrophobicity. Another factor that may contribute to the inhibition is the polarizability of the anion. Anions are more polarisable than cations of the same charge density [64], which explains their dominating effect on enzymes [26]. Experimental values of electronic polarizability obtained through dielectric measurements on gaseous isolated molecules show that acetic acid has less polarizability (5.15 Å³) than propanoic acid (6.96 Å³) [65]. Thus, inhibition of TfXYN10A xylanase appears to follow an increase in polarizability of the anion. The correlation of increased enzyme inhibition with increasing hydrophobicity and polarizability of the anion was also observed in the activity of tyrosinase in ILs solutions [34]. The propionate-based ILs used in this study exhibit lower viscosity compared to acetate-based ILs [45], which may contribute to the observed higher enzyme inhibition. Xu (2017) found that viscosity decreases as temperature increases due to the weakening interaction between cation and anion [66].

In dilute IL solutions kosmotropic anions favour enzyme functioning, while chaotropic anions do not favour it [25]. Propionate is more kosmotropic than acetate [67], but more inhibiting for the enzyme, which indicates that inhibition cannot be explained by the kosmotropicity effect of the anions. This result agrees with previous studies showing that the anion effect does not always follow the Hofmeister sequences [26].

Enhanced size and hydrophobicity (presence of a benzoic ring) and the polarizability (12.07 Å³ in solid state [65]) of guaiacolate may account for the stronger enzyme inhibition of this anion compared to acetate and propionate anions at 5% IL concentrations. It appears that

an increase in hydrophobicity of the anion decreases the IL concentrations needed to inactivate the enzyme. According to Zhao (2016) [25], in diluted aqueous IL solutions (high concentration of water) kosmotropic anions bearing high H-bond basicity (acetate, propionate) tend to interact strongly with water molecules and become enzyme-friendly [25]. As the anion becomes more hydrophobic (guaiacolate), it is less hydrated and tends to interact with the enzyme. This interaction may be stronger due to enhanced polarizability of this anion and may lead to inactivation of the enzyme at lower IL concentrations.

Based on an examination of the protein structure, Chawachart et al. (2014) [40] proposed a reason for the difference in IL inhibition between GH10 and GH11 xylanases. They proposed that the narrow and deep active site of GH11 xylanases allows transient binding of large amounts of [EMIM]⁺ cations, whereas, in the more open active site of GH10 xylanases, the binding of [EMIM]⁺ cations is distributed in a more restricted area [40]. We observed in this study that the active site of GH10 xylanase TfXYN10A has separate cation and anion binding sites (Fig. 6). It is likely that together with the high combined hydrophobic binding surface of guaiacolate and cation, the charge interaction between the anion and cation contribute to the synergistic effect of cation and anion in binding to the active site, thus inhibiting more strongly enzyme activity. This effect is smaller in propionate and acetate due to their weaker binding to the active site (Table S1). Accordingly, no kinetically trapped acetate was observed in the simulation of Jaeger and Pfaendtner with the GH11 xylanase in the presence of [EMIM]OAc [44].

While in GH10 xylanase TfXYN10A, the cation and anion binding sites seem to be in separate active site areas (Fig. 6), in GH11 xylanase DtXYN11B, the cations and anions seem to bind quite much to the same areas (Fig. 7). Since cation binding energies are higher than guaiacolate binding energy, then cation binding might possibly be preferred in the active site

of DtXYN11B (Table S1). These kind of differences between different enzymes may fine tune how the combined dynamic effect of cation and anion interactions with the enzyme cause enzyme inhibition. Apparently, stronger binding of guaiacolate to TfXYN10A than to DtXYN11B (Table S1) may explain why the inhibition with guaiacolates was strongest with GH10 xylanase. The double ring in the cations and the single ring in guaiacolate form larger hydrophobic binding surfaces. The active site of glycosyl hydrolases contains hydrophobic surfaces to bind the hydrophobic parts of sugar rings in the carbohydrate chains. These surfaces would also form strong interactions with the hydrophobic parts of IL cations and anions.

In conclusion, the shape of the active site, overall interaction properties of the substrate binding area and the interaction mode between the enzyme and inhibitor appear to play an important role in determining the sensitivity of the enzyme to competitive inhibition caused by IL molecules typically binding quite close to the catalytic residues. Both cation and anion contribute to the inhibition by competing out the substrate for binding to the active site. The enzyme-specific differences in IL-tolerance were shown both by experimental and modelling studies. Thus, the inhibition effect depends on the combined properties of the enzymes and ILs. The obtained principles can be used to plan process conditions for enzyme treatments in ILs, to design enzyme-friendly ILs for biotechnical use and to engineer new and more IL-tolerant enzymes.

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Figure legends

Fig. 1. Comparison between the activities of *D. thermophilum* GH11 xylanases (DtXYN11B and DtXYN11B-DS) and *T. flexuosa* GH10 xylanase (TfXYN10A) in 15% ILs with 1% beechwood xylan. Activities were measured at 70°C and pH 6.5 for DtXYN11B and DtXYN11B-DS and pH 6 for TfXYN10A. The relative activity is shown in relation to a 100% activity level without the IL.

Fig. 2. Activities of *T. flexuosa* GH10 xylanase (TfXYN10A) and *D. thermophilum* GH11 xylanases (DtXYN11B and DtXYN11B-DS) in 5% guaiacolate-based ILs with 1% beechwood xylan. Activities were measured at 70°C and pH 6 for TfXYN10A and pH 6.5 for DtXYN11B and DtXYN11B-DS. 100% activity is the activity level without the IL.

Fig. 3. Activity of *T. flexuosa* GH10 xylanase (TfXYN10A) in 1% and 4% substrate in the presence of 15% ILs. Activity was measured at 70°C and pH 6. 100% activity is the activity in each substrate concentration without IL.

Fig. 4. Activity of *T. flexuosa* GH10 xylanase (TfXYN10A) in 1% and 4% substrate in the presence of 5% guaiacolate-based ILs. Activity was measured at 70°C and pH 6. 100% activity is the activity in each substrate concentration without IL.

Fig. 5. Activity of *T. flexuosa* GH10 xylanase (TfXYN10A) in 5–35% ionic liquids with 1% beechwood xylan as substrate. Activity was measured at 70°C and pH 6. 100% activity is the activity without IL.

Fig. 6. Docking of 1,5-Diazabicyclo[4.3.0]non-5-enium [DBNH]⁺ cation and guaiacolate anion to the active site of *T. flexuosa* GH10 xylanase (TfXYN10A). From separate dockings of these ligands, two [DBNH]⁺ cations (marked by +) and one guaiacolate anion (marked by -) that have space to fit the active site canyon simultaneously were chosen to be shown. The

upper [DBNH]⁺ cation had the lowest ΔG among the [DBNH]⁺ poses. Corresponding [DBNH]⁺ clusters are shown in Fig. S17C and Fig. S18A and S18B. The catalytic residues (one with negative charge) are located below the cations. Nitrogens are shown in blue and oxygens in red.

Fig. 7. Docking of IL molecules to *D. thermophilum* GH11 xylanase (DtXYN11B). Positions of binding guaiacolate anions (A), 1,5-Diazabicyclo[4.3.0]non-5-enium [DBNH]⁺ cations (B) and 1,1,3,3-Tetramethylguanidinium [TMGH]⁺ cations (C) are shown. From separate SwissDock runs, the cation/anion molecules that could fit the active site simultaneously are shown for each ligand after placing the highest energy binder (shown by star). Fig S15 shows the corresponding clusters. The catalytic residues are shown in grey and their oxygens in red.

738 **Table 1.** The kinetic parameters of TfXYN10A with ionic liquids.

Ionic liquid	Relative $V_{\max}$	K_M (mg/mL)	Relative $V_{\max} / K_M$
No IL	1.000 ± 0.022	0.506 ± 0.048	1.986 ± 0.197
[DBNH]OAc	1.002 ± 0.016	0.651 ± 0.044	1.544 ± 0.094
[DBNH]CO ₂ Et	1.267 ± 0.011	0.650 ± 0.023	1.951 ± 0.076
[DBUH]OAc	1.051 ± 0.010	0.735 ± 0.025	1.431 ± 0.048
[DBUH]CO ₂ Et	1.072 ± 0.015	0.641 ± 0.021	1.672 ± 0.057
[EMIM]OAc	1.076 ± 0.023	0.729 ± 0.060	1.480 ± 0.121
[mDBN]Me ₂ PO ₄	1.138 ± 0.000	0.657 ± 0.001	1.732 ± 0.003
[TMGH]OAc	1.037 ± 0.052	0.625 ± 0.088	1.673 ± 0.181
[TMGH]CO ₂ Et	1.034 ± 0.026	0.695 ± 0.050	1.492 ± 0.072
[DBNH]guaiacolate	0.357 ± 0.025	1.284 ± 0.217	0.281 ± 0.027
[DBUH]guaiacolate	0.475 ± 0.020	2.928 ± 0.472	0.164 ± 0.023
[TMGH]guaiacolate	0.324 ± 0.003	11.335 ± 0.346	0.028 ± 0.000

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