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Characterization of supercharged cellulase activity and stability in ionic liquids

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ABSTRACT

Ionic liquids (ILs) have many potential benefits in biochemical processes, including improved substrate or product solubility, increased enzyme selectivity, and higher yield. Varying ion substituents allow ILs to be tuned or optimized to achieve a specific goal. Unfortunately, optimization based on a single design criterion can have undesirable side effects on other process components. For example, hydrophilic ILs capable of efficiently dissolving biomass often inhibit enzymatic activity during hydrolysis. A panel of nine different aqueous ILs was selected for this study to systematically assess which factors contribute to the loss of enzyme activity. The activity of endoglucanase E1 from *Acidothermus cellulolyticus* steadily decreased in higher concentrations of ILs, especially in the presence of the common cellulose dissolving solvent 1-ethyl-3-methylimidazolium acetate. The impact of most other ILs could be rationalized via the Hofmeister series. Enzyme behavior was further probed by rationally modifying the surface charge of E1. Variants were computationally designed to have positively or negatively charged surfaces and assessed for activity in ILs. Surprisingly, positive supercharging maintained wild type activity levels in ILs, while negative supercharging drastically reduced activity. Discrepancy between stability and activity measurements for some ILs indicated active site inhibition or other unique inactivation mechanisms might be crucial components to consider in future studies.

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1. Introduction

Salts with melting temperatures below 100 °C, termed ionic liquids (ILs), offer a variety of benefits in industrial processes. Much like organic solvents, ILs can be used to adjust polarity or hydrophobicity in a reaction system and alter the solubility of substrates and products [1]. ILs can also enhance stereoselectivity or facilitate phase separation [2]. Among other applications, ILs have been proposed as a method for pretreating cellulosic biomass prior to enzymatic hydrolysis. Pioneering work by Rogers and coworkers first demonstrated that neat ILs could dissolve cellulose [3], and many imidazolium-based ILs have since been tested on lignocellulosic substrates [4]. ILs that have performed exceptionally well with industrially relevant substrates include 1-ethyl-3-methylimidazolium acetate ([Emim]OAc), 1-butyl-3-methylimidzolium chloride ([Bmim]Cl), 1-allyl-3-methylimidzaolium chloride ([Amim]Cl), and 1,3-dimethylimidazolium dimethylphosphate ([Dmim]DMP) [4].

Unfortunately, it remains difficult to find an IL that dissolves biomass without detrimentally impacting enzyme activity. The high hydrogen bond basicity, or thermodynamic tendency to act as a hydrogen bond acceptor, typically makes cellulose-dissolving ILs incompatible with enzymes [5]. Early studies indicated cellulase activity was drastically reduced in ILs [6]. In recent years, numerous other enzymes have been assessed for activity and stability across a wide range of ILs [4,7–10]. Various strategies have been proposed for preserving enzymatic activity [9,11] but a generic stabilization approach remains elusive.

As early as the 19th century, the role of ions in stabilizing or destabilizing proteins was categorized via the Hofmeister series [12]. Enzymes typically prefer large, low charge density, kosmotropic anions over small, high charge density, chaotropic anions [7,8,13,14]. This trend is reversed for cations due to the "match-

Abbreviations: E1, endoglucanase 1; pE1, superpositive E1; nE1, supernegative E1; pNPC, p-nitrophenol-β-D-cellobioside; IL, ionic liquid; [Emim], 1-ethyl-3-methylimidazolium; [Bmim], 1-butyl-3-methylimidazolium; [Amim], 1-allyl-3-methylimidzaolium; [Dmim], 1,3-dimethylimidazolium; BF_4, tetrafluoroborate; DMP, dimethylphosphate; DEP, diethylphosphate; DBP, dibutylphosphate; NO_3, nitrate; OA, cacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, *N*-Cyclohexyl-2-aminoethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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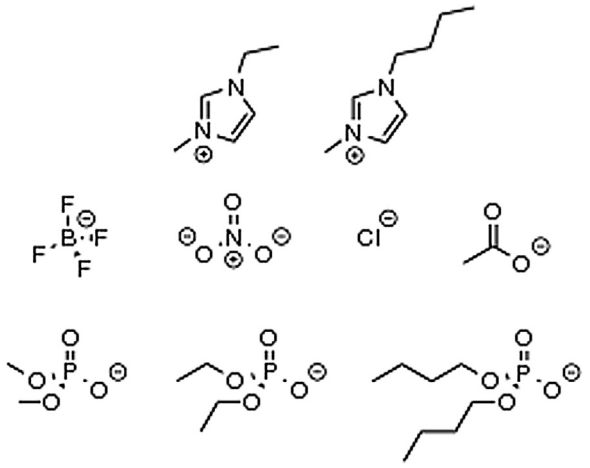

Fig. 1. Ionic liquids used in experimental assays.
Top row, left to right: cations 1-ethyl-3-methylimidazolium ([Emim]) and 1-butyl-3-methylimidazolium ([Bmim]). Middle row: anions tetrafluoroborate (BF_4), nitrate (NO_3), chloride (Cl), and acetate (OAc). Bottom row: anions dimethylphosphate (DMP), diethylphosphate (DEP), and dibutylphosphate (DBP).

ing kosmotropicity" effect [15]. Although the Hofmeister series provides useful guidance, examples exist where this classification scheme fails to apply [7]. In addition to ion kosmotropicity, solvent properties such as viscosity, polarity, hydrophobicity, nucleophilicity, or hydrogen bond basicity can be important [8,16–18]. To date there is little consensus regarding how water, IL, and enzymes interact in aqueous-IL solutions. Solvated ions can influence enzymes in a variety of ways, include stripping of surface water molecules, changing of protein structure and dynamics, modification of surface pH, or preferential stabilization of the unfolded state [1,7,19,20].

Modifying the surface charge of a protein inherently influences its stability, solubility, and aggregation propensity [21–23] and is likely to play a key role in moderating IL-enzyme interactions. Kaar and coworkers experimentally demonstrated the importance of electrostatic interactions by chemically modifying surface charge groups on cellulase, lipase, papain, and chymotrypsin [24–26]. Chemical modification via acetylation or succinylation improved resistance against denaturing ILs [Bmim]Cl and [Emim]ethylsulfate. Furthermore, molecular dynamics simulations indicated surface charge significantly altered ion interactions at the enzyme surface [27]. Other reports have noted a correlation between native enzyme charge and halotolerance [28–30], providing additional evidence that electrostatic interactions can strongly influence protein behavior in ionic solutions.

Systematically altering protein charge via mutagenesis could provide a direct route for probing the IL-enzyme interface. Although the "supercharging" concept introduced by Liu and coworkers [31] has not previously been employed in conjunction with ionic liquids, it provides an extreme example for studying enzyme charge modification. Supercharging imparts a high net charge, either positive or negative, on the protein surface [21–23]. Although successful in many regards, supercharged variants can display decreased binding affinity or loss of enzymatic activity [31]. Ideally, careful selection of charged mutation sites minimizes these negative impacts.

To build upon previous supercharging studies, we set out to explore IL inactivation mechanisms for supercharged variants of endoglucanase (E1) from *Acidothermus cellulolyticus*. E1 is an industrially relevant enzyme with high native stability that has been well characterized through mutagenesis and structural studies [32–39]. A single mutation near the active site (Tyr245Gly) has previously been shown to reduce product inhibition [32] and was therefore included in wild type and design sequences. Positively charged (pE1) and negatively charged (nE1) variants were computationally designed to have altered surface electrostatics. Each enzyme variant was tested against a panel of imidazolium-based ILs (Fig. 1) in aqueous solutions to assess specific ion interactions.

2. Materials and methods

2.1. Computational protein design

The computational method used to selectively introduce charged mutations on the surface of E1 closely resembled the Rosetta approach demonstrated by the Kuhlman lab [40,41]. Candidate mutation sites were selected based on solvent exposed surface area calculations [42] and evaluated using the Rosetta energy function [43] as implemented in SHARPEN [44]. Key catalytic residues (Glu162/Glu282), binding residues conserved within glycoside hydrolase family 5 (Arg62, His116, Asn161, His238, Tyr240, Trp319), disulfide bonds (Cys34/Cys120, Cys168/171), and prolines were held fixed and not allowed to mutate during the design process. Either positively charged (Lys, Arg) or negatively charged (Glu, Asp) mutations were allowed at each design site, and a selective energy bias was applied to adjust the number of mutations and achieve a desired net charge. Based on previous supercharging reports [31,40,41], a net change in charge of ~20 e^- units was targeted for E1 designs. Supernegative nE1 contained a total of 24 mutations (93.3% sequence identity to wild type E1), of which 13 were initially polar, 6 were nonpolar, 2 were positively charged, and 3 were negatively charged (Asp to Glu mutations). For pE1, 26 candidate mutation sites were identified. Site 165 had previously been characterized as unfavorable for positively charged mutations [33] and was therefore left as wild type aspartic acid. In addition, site 324 was deemed too close to the active site upon visual inspection and was left as wild type aspartic acid. Therefore, the final pE1 design contained 24 mutations (93.3% sequence identity to wild type E1), of which 13 were initially polar sites, 6 were nonpolar, 1 was negatively charged, and 4 were positively charged (Lys to Arg mutations). More than half (14 of 24) of the mutation sites were common to both pE1 and nE1. The full design sequences are available in Supp. Info.

2.2. Enzyme expression and purification

Supercharged sequences were constructed via commercial gene synthesis (DNA2.0, California, USA). Expression and purification steps followed those previously reported [39]. Briefly, proteins were expressed in *E. coli* BL21 (DE3) pLySs cells using a glucose/lactose induction system, lysed using sonication, and purified via immobilized metal affinity chromatography. Soluble protein yields ranged from 10 to 30 mg protein per liter culture. All variants except pE1 were buffer exchanged into 50 mM sodium acetate pH 5; pE1 was buffer exchanged and stored in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5 for improved stability. Proteins were assessed for relative size and purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 3).

2.3. Activity and stability assays

Expressed enzymes were tested for activity using the fluorescent substrate p-nitrophenol-β-D-cellobioside (pNPC). Assays were performed at 50 °C for 30 min in 125 mM sodium acetate pH 5 with 200 ug pNPC. Similarly, salt and IL assays were performed at 50 °C, pH 5 for 30 min. Retained activity (T_{50}) was measured after a 30 min preincubation temperature ranging from 40 to 80 °C. Curves were fit to T_{50} data using an inverse sigmoidal dose-response curve, and T_{50} was defined as the temperature that

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