Critical Assessment of Structure-based Approaches to Improve Protein Resistance in Aqueous Ionic Liquids by Enzyme-wide Saturation Mutagenesis

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1 Abstract

Ionic liquids (IL) and aqueous ionic liquids (aIL) are attractive (co-)solvents for green industrial processes involving biocatalysts, but often reduce enzyme activity. Experimental and computational methods are applied to predict favorable substitution sites and, most often, subsequent site-directed surface charge modifications are introduced to enhance enzyme resistance towards aIL. However, almost no studies evaluate the prediction precision with random mutagenesis or the application of simple data-driven filtering processes. Here, we systematically and rigorously evaluated the performance of 22 previously described structure-based approaches to increase enzyme resistance to aIL based on an experimental complete site-saturation mutagenesis library of BsLipA screened against four aIL. We show that, surprisingly, most of the approaches yield low gain in precision (GiP) values, particularly for predicting relevant positions: 14 approaches perform worse than random mutagenesis. Encouragingly, exploiting experimental information on the thermostability of BsLipA or structural weak spots of BsLipA predicted by rigidity theory yields GiP = 3.03 and 2.39 for relevant variants and GiP = 1.61 and 1.41 for relevant positions. Combining five simple-to-compute physicochemical and evolutionary properties substantially increases the precision of predicting relevant variants and positions, yielding GiP = 3.35 and 1.29. Finally, combining these properties with predictions of structural weak spots identified by rigidity theory additionally improves GiP for relevant positions up to 4-fold to ~10 and sustains or increases GiP for relevant positions, resulting in a prediction precision of ~90% compared to ~9% in random mutagenesis. This combination should be applicable to other enzyme systems for guiding protein engineering approaches towards improved aIL resistance.

2 Introduction

With the world population continuing to increase [1], studies forecast a shortage of natural resources, such as fresh water [2] and fossil fuels [3, 4]. Green industrial processes, such as the enzymatic production of biofuel and other valuable products from abundantly available plant material, attempt to solve these problems [5-11]. However, in particular, current biofuel production uses environmentally unfriendly acid catalysis and requires large amounts of freshwater for the reaction workup [12-14]. Consequently, environmentally friendly alternatives to produce biofuel are needed. Ionic liquids (IL) are attractive solvents for this, as some IL dissolve cellulosic plant material without the need for heat activation or pretreatment using solvents such as strong acids or carbon disulfide [12, 15]. While pure IL often result in enzyme activities impractical for industrial processes [16-20], IL-pretreated holocellulose retains a high digestibility for enzymes after recrystallization in water [21]. Aqueous ionic liquids (aIL), e.g., the remnants of IL in recrystallized holocellulose, show a reduced yet still marked impact on enzymatic activity [22, 23]. Hence, for using aIL in green industrial processes, it is of utmost importance to understand how aIL affect enzyme stability and activity and to use this knowledge to improve enzyme resistance against these solvents.

To improve enzyme resistance to aIL, studies frequently relied on straightforward and well-established approaches, such as directed evolution [24, 25], to generate aIL-resistant enzyme variants [26-28]. The low experimental efforts, however, come with the drawback that mutations are randomly generated (albeit this can be directed to a certain degree using, e.g., modified polymerases), leading predominantly to minor changes in the protein [29] and often incomplete coverage of the sequence and position space [30]. More recently, approaches to increase aIL resistance transposed towards data-driven protein engineering approaches, which rely on prior knowledge to improve specific enzyme properties by introducing changes at distinguished sequence positions and can cover the whole sequence space. Here, variant libraries are designed by predicting advantageous positions based on, e.g., structure [31-35] or consensus information [36-39] or by predicting substitutions (exchanges of an amino acid to a different amino acid due to a mutation in the corresponding DNA sequence) at distinct positions with a specific goal in mind, e.g., in disulfide bond engineering [40] or surface charge modification approaches [17-20, 41-44]. Surface charge modification, in particular, is a widely proposed approach to increase aIL resistance following the rationale that introducing charged, ion-repelling substitutions at the protein surface can prevent aIL interactions with enzymes and their subsequent effects [17-20, 41, 44-46]. Over the years, this approach became noticeably more specific, as it evolved from a global chemical modification of all lysine residues of a protein [18-20] over fractional substitutions of lysine residues [41] to an NMR-based site-specific approach targeting distinguished positions around perturbed protein residues [17]. However, the lack of available, systematic large-scale data prevented evaluating the performance of such approaches against random mutagenesis or simple structure-based guidelines.

For the model enzyme *Bs*LipA, a complete site-saturation mutagenesis library (termed "*Bs*LipA SSM library" hereafter) is available that covers all 3620 potential single substitutions with natural amino acids (181 substitution sites with 20 possible substitutions at each site) [16]. The *Bs*LipA SSM library was screened towards thermostability [33], resistance to four detergents [33, 47] resistance to three organic solvents [30], and aIL resistance to four imidazolium-based aIL (0.9 M   1-butyl-3-methylimidazolium   bromide   ([BMIM/Br]),   1.2 M   1-butyl-3-

methylimidazolium chloride ([BMIM/Cl]), 0.6 M 1-butyl-3-methylimidazolium iodide ([BMIM/I]) and 0.7 M 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([BMIM/TfO])) [16]. The concentrations of the individual aIL were chosen to result in residual activities of 30-40% with respect to the activity in buffer to allow for relative comparisons between the aIL [16]. BsLipA is particularly interesting for that, as it is a small lipase and does not show interfacial activation, but has often been used in similar experimental and computational studies [16, 17, 33, 43-45, 48, 49], and high-resolution X-ray crystal structures (PDB ID: 1I6W [50] and 1ISP [51]) are available.

An initial analysis of the BsLipA SSM library showed that more than half of all amino acid positions contribute to IL resistance of BsLipA. It further revealed substitution patterns at which presumably high fractions of aIL-resistant variants occur, e.g., for substitutions at specific secondary structure elements [16] or substitutions to chemically different amino acids [16]. Subsequent studies based on the BsLipA SSM library proposed surface charge-engineering and increasing the substrate cleft polarity to improve aIL resistance [16, 45, 49]. However, in these cases, the results were not related to *a priori* probabilities, such that the performance of these guidelines for suggesting aIL-tolerant variants may be overrated (see also below).

A previous large-scale analysis of the BsLipA SSM library with respect to thermostability and detergent resistance revealed significant improvements in prediction accuracy compared to random mutagenesis for a data-driven structural stability-based approach [33]. Additionally, data mining of the BsLipA SSM library [30, 33] and another large-scale library [52] showed that applying simple physicochemical properties to predict substitutions, such as the solvent-accessibility (SA) or the change in unfolding free energy ($\Delta\Delta G_{unf}$), increases the prediction accuracy for thermostability or detergent resistance [30, 33, 52]. Hence, the BsLipA SSM library offers a unique opportunity to evaluate the performance of commonly applied approaches to increase aIL resistance towards their prediction accuracy of beneficial substitutions and substitution sites. Furthermore, the BsLipA SSM library can be used to systematically evaluate new guidelines aiming at a time- and cost-efficient knowledge-driven protein engineering towards aIL resistance.

In this work, we show for the BsLipA SSM library that the prediction accuracy of commonly used approaches and guidelines to improve aIL resistance of enzymes is surprisingly low. We apply rigorous binary classifiers and report the results relative to performing unbiased random mutagenesis for evaluation. This way, we account for *a priori* probabilities. Furthermore, we introduce a rational approach that outperforms currently applied approaches, can be computed within a few hours, and only requires a protein structure as input.

3 Results

3.1 In Total, 9% of All Variants Show Significantly Increased aIL Resistance, and 57% of All Positions Harbor Such Variants

In total, the BsLipA SSM library contains 3620 variants at 181 positions that were tested for residual activity (RA$_{aIL}$; **Eq. S1**) in 0.9 M [BMIM/Br], 1.2 M [BMIM/Cl], 0.6 M [BMIM/I], and 0.7 M [BMIM/TfO] and subsequently assessed concerning the variance of the data and significance of changes (see **section 3.1** in Supplementary Information) [16]. The aIL resistance

of a variant was considered significantly improved when $RA_{variant,aIL} \geq RA_{wildtype,aIL} + 3\ \sigma_{aIL}$, with $RA_{variant,aIL}$ and $RA_{wildtype,aIL}$ being the RA_{aIL} of the variant in aIL and buffer, respectively, and σ_{aIL} being the standard deviation of the assay in the respective aIL [16]. $3\ \sigma_{aIL}$ was chosen because it corresponds to a *p*-value below 0.01, assuming a Gaussian distribution of the RA_{aIL}. Throughout this study, variants with significantly improved aIL resistance and positions harboring such substitutions will be termed "relevant variants" or "relevant positions". A graphical representation of the *Bs*LipA variant distribution of variants and positions is shown in **Figure 1**.

Averaged over all four aIL, only 9% of all substitutions (310 variants) yielded relevant variants ([BMIM/Br]: 8% or 263 variants; [BMIM/Cl]: 13% / 462; [BMIM/I]: 6% / 206; [BMIM/TfO]: 9% / 292). This proportion (9%) represents the chance of finding relevant variants using unbiased random mutagenesis, e.g., by error-prone PCR (epPCR) with equal probabilities for all variants; experimental biases, such as the preference of *Taq* polymerase [53] in epPCR for AT → GC transitions, are thus not considered [54]. This value will subsequently be used in our analyses to evaluate the performance of approaches to predict relevant variants. The percentage of relevant variants is comparable to that obtained for detergent resistance (~12%) [33, 47]. The slightly lower percentage for aIL may be due to using $3\ \sigma_{aIL}$ as a limit to define significance, whereas $2\ \sigma_D$ was used in the case of detergents [33, 47]. The conservative limits are used to counterbalance experimental uncertainties in the RA_{aIL} that originate from enzyme activities measured in the supernatant [16], which may be influenced by differences in thermodynamic or kinetic protein stability [34, 55] or protein expression [56]. The RA_{aIL} distributions for the four aIL are shown in **Figure S1**.

In contrast, more than half of all substitution sites (57% or 103 positions) harbored such relevant variants ([BMIM/Br]: 50% or 91 positions; [BMIM/Cl]: 69% / 124; [BMIM/I]: 52% / 95; [BMIM/TfO]: 57% / 104). Interestingly, almost half of all BsLipA positions (89 positions) yield relevant variants in three or more aIL, and only ~20% (39 positions) yield variants that are not improved in any aIL (Figure 1C/D).Thus, more than twice the number of positions in *Bs*LipA yield relevant variants compared to detergent resistance (~27%) [33]. This proportion (57%) represents the chance of finding a position that harbors relevant variants using unbiased random mutagenesis and will subsequently be used in our analyses to evaluate the performance of approaches to predict relevant positions. Here, the majority of the positions yields less than one aIL resistant variant, and few positions yield multiple (> 4) aIL resistant variants (**Figure 1A/B**). Hence, in *Bs*LipA, each of the 103 relevant positions yields on average three relevant variants out of 20 possible substitutions. When using unbiased random mutagenesis, the experimental effort to identify 10 unique relevant variants or positions on average sums up to screening ~117 and ~18 variants, respectively.
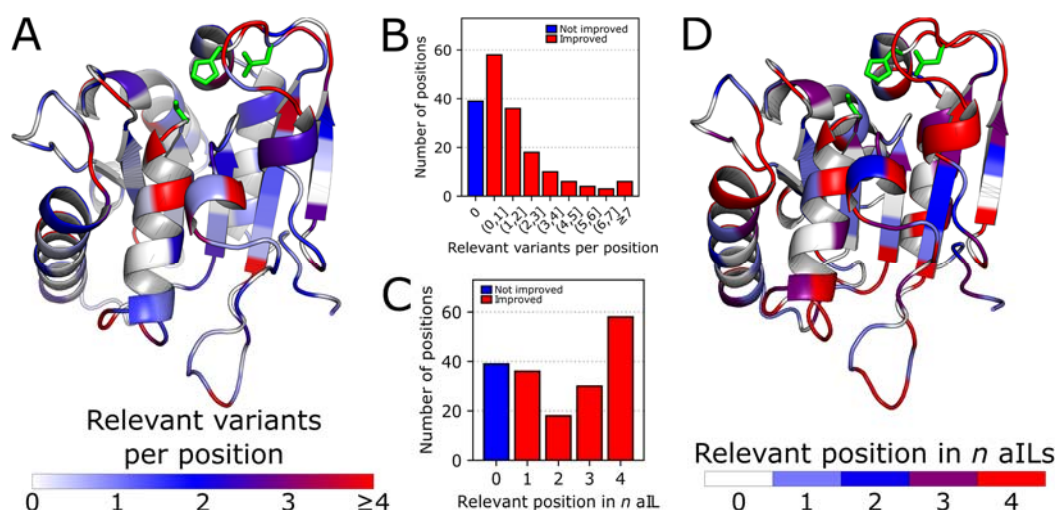
Figure 1: Distributions of relevant variants and positions for the four aIL in the *Bs*LipA SSM library. Data is analyzed by focussing on relevant variants (A-B) and relevant positions (C-E). (**A**) The average number of relevant variants per position is mapped onto the *Bs*LipA structure with blue (red) color depicting a low (high) amount of variants per position. The catalytic site residues S77, D133, and H156 are depicted as sticks and colored in green. (**B**) Average number of relevant variants per position. The majority of the positions yields less than one aIL resistant variant, and few positions yield multiple (> 4) aIL resistant variants. (**C**) Number of positions that are relevant in $n = 0$ to 4 aIL. Almost half of all *Bs*LipA positions (89 positions) yield relevant variants in three or more aIL, and only ~20% (39 positions) yield variants that are not improved in any aIL. (**D**) Data of (**C**) mapped onto the *Bs*LipA structure with colors depicting the number of aIL (white: 0; light blue: 1; blue: 2; magenta:3; red:4). The catalytic site residues S77, D133, and H156 are depicted as sticks and colored in green.

3.2 Definition of Measures for Evaluating the Predictive Power of Approaches

We defined two measures to evaluate the performance of a given approach for improving aIL resistance on the *Bs*LipA SSM library based on binary classification: the gain-in-precision (GiP, **Eq. S3**, [57]) on a variant-wise level (GiP$_{var}$) and the gain-in-precision on a positional level (GiP$_{pos}$). The GiP$_{var}$ and GiP$_{pos}$ describe the relative likelihoods to correctly predict relevant variants or relevant positions compared to unbiased random mutagenesis. Note that GiP is not affected by data prevalence and data imbalance, in contrast to other measures of binary classification, such as accuracy [58], which is important in view of the underrepresentation of relevant variants. Note, too, that we focus on precision and not recall [57] because, for our application, it is more important to have a high fraction of correctly classified instances among those classified relevant than to have high coverage of the relevant class: Substantially improved enzyme variants often incorporate only a few (1-3) substitutions [59-62], and additional substitutions do not easily lead to further improvements, particularly when they are interacting[25]. This is because the majority of substitutions destabilize an enzyme, limiting the way how substitutions are combinable [46, 59, 63-66]. Furthermore, despite state-of-the-art high-throughput selection [67-70] and screening [71-73] techniques, protein engineering approaches are still limited to a small number of positions if the whole sequence space shall be investigated, as the library size increases exponentially (combining all possible substitutions at, e.g., six positions

already leads to $20^6 = 6.4 \cdot 10^7$ variants) [74]. Hence, identifying a few relevant variants and positions is necessary *and* sufficient for most protein engineering approaches.

Because our analysis is focused on general applicability towards several aIL instead of individual solvents, the GiP values are averaged over the four aIL of the *Bs*LipA SSM library. Yet, to provide an estimate of the data variance, the ranges of the numbers of relevant variants and positions, and the relations to the total considered variants and positions, are presented across the four aIL (**Table 1**, **Table S1**). Finally, we performed a Boschloo's 'exact'-test to determine if the observed populations of relevant variants and positions of a given approach were significantly different ($p \leq 0.05$) from those of random mutagenesis [75]. Here, we assessed the *p*-value of the test statistics regarding the populations of relevant variants or positions versus not-relevant variants or positions compared to random mutagenesis, which describes the probability of finding a sample statistic as extreme as the test statistics. Unless all *p*-values are < 0.05, the lowest and highest *p*-values observed over the four aIL are shown in **Table 1** and **Table S1**.

3.3 Assessment of Commonly Applied Approaches to Improve aIL Resistance

We extracted 22 approaches to improve enzyme resistance towards aIL from the literature and evaluated their performance to improve aIL resistance using the above-defined measures (**Table S1**). These approaches can be classified into six groups (**Figure 2**):

I) approaches that determine relevant positions from experimental structural data for the system (**A1-A5**);

II) approaches that determine relevant positions from extensive computations (**A6-A11**);

III) approaches that determine relevant positions from experimental biochemical data on other "environmental" effects, such as temperature or solvents with detergents (**A12-A13**);

IV) one approach where relevant positions are determined as structural weak spots by rigidity theory without considering specific aIL effects (**A14**);

V) approaches that modify surface charges (**A15-A20**);

VI) two approaches that did not consider *a priori* information (**A21-A22**).
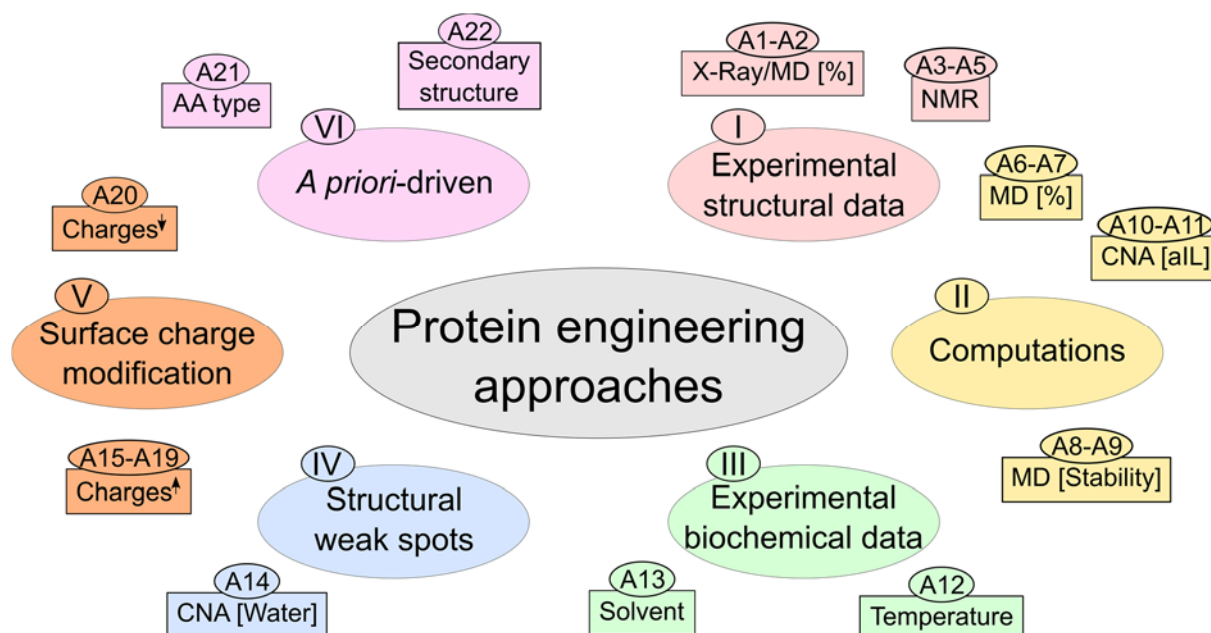
Figure 2: Overview of evaluated structure-based approaches described in the literature for improving aIL resistance. The classification of the approaches (I-VI) is described in the text. Most approaches rely on analyzing direct protein-aIL interactions (**A1-A7**), whereas only a few investigate subsequent effects of the aIL interactions on the protein (**A8-A11**).

We will summarize the results for the approaches of each group here (**Table 1**). For detailed information on each approach, see the Supporting Information and **Table S1**.

Group I: Approaches **A1** and **A2** used binding sites identified from X-ray crystal structures of *Bs*LipA in the presence of aIL, which were subsequently refined by molecular dynamics (MD) simulations. Approaches **A3**-**A5** used two-dimensional $^{15}N/^{1}H$ HSQC NMR experiments to identify positions that experienced perturbations in their local chemical environment upon incubation in [BMIM/Cl]. In both cases, a similar number of relevant sites (23 to 24) were predicted. These sites overlap to a low degree between the approaches (26% of **A1**-**A2** sites are found in **A3**-**A5** and 25% vice versa), but to a high degree with the *Bs*LipA SSM library reference data (74% and 80%). For specific changes to charged amino acids, in either subgroup, high GiP$_{var}$ values of ~2.2 to 2.5 are associated with low GiP$_{pos}$ values (~0.3 to 0.5), indicating that such charge changes are effective at the predicted relevant sites with ~1/3 to ½ of the precision of random mutagenesis only. In turn, moderate GiP$_{var}$ (≤ 1.8) and GiP$_{pos}$ (≤ 1.2) values are obtained if substitutions to all amino acids are evaluated. I.e., GiP$_{var}$ = 1.79 and GiP$_{pos}$ = 1.2 in approach **A2** indicate that ~65 and ~15 variants have to be screened to obtain ten relevant variants and positions, respectively, compared to ~118 and ~18 when using random mutagenesis.

Group II: In approaches **A6** and **A7**, we identified aIL binding sites of *Bs*LipA from extensive MD simulations using distance-based interaction criteria and evaluated the 20 most occupied positions for each solvent [43]. The identified sites showed a low to moderate overlap with binding sites of **A1**-**A2** (41% and 26% for **A6** and **A7**, respectively) and **A3**-**A5** (18% and 21% for **A6** and **A7**, respectively), but a high overlap with the *Bs*LipA SSM library reference data (73% and 85%). However, similar to **A1** and **A3**-**A4**, specific changes to positively (negatively) charged amino acids for cation (anion) binding sites yielded moderate GiP$_{var}$ of ~1.3 but substantially

lower GiP$_{pos}$ values (~0.3), again indicating a low precision for such predicted relevant sites compared to random mutagenesis in the context of charge changes.

In approaches **A8-A11**, we assessed whether predictions based on aIL-induced local structural stability changes, identified using either MD simulations (**A8-A9**) or the rigidity theory-based Constraint Network Analysis (CNA) (**A10-A11**), lead to increased GiP values. Introducing charged amino acids at solvent-exposed positions (**A8** and **A10**) yielded high GiP$_{var}$ ≈ 2 but again low GiP$_{pos}$ ≈ 0.75 values. In turn, considering substitutions to all amino acids at positions irrespective of the solvent exposure (**A9** and **A11**) yields moderately increased GiP$_{var}$ (~1.35) and GiP$_{pos}$ (~1.15) values. Notably, the results are comparable to predictions from **A1-A5**, indicating that computational approaches predict relevant variants and positions with similar precision as experiment-based ones without the need for cost- and time-intensive experiments.

Group III: Approaches **A12** and **A13** probe to what extent knowledge of relevant positions gained from optimizing *Bs*LipA against temperature or detergent influence can be transferred to increasing aIL resistance. In the latter case (**A13**), only moderate GiP$_{var}$ (1.56) and GiP$_{pos}$ (1.20) are obtained. In the former case (**A12**), however, the highest GiP$_{var}$ = 3.03 and GiP$_{pos}$ = 1.61 of all tested approaches are obtained.

Group IV: Approach **A14** assesses whether structural weak spots of the *Bs*LipA structure identified with the rigidity theory-based Constraint Network Analysis method are relevant positions. Contrary to **A11**, weak spots were identified based on structural ensembles of *Bs*LipA generated in water only and determined from phase transitions upon thermal unfolding. With this approach, the highest GiP$_{pos}$ = 1.41 among all evaluated computational approaches is obtained, and the fifth-highest GiP$_{var}$ = 2.39 among all evaluated experimental and computational approaches. Note that for groups III and IV, the number of predicted relevant positions is low (6 to 11), which facilitates identifying beneficial substitution combinations later. However, the number of variants is still high because we evaluate substitutions to all amino acids. Hence, further rules are needed to limit the substitution possibilities (see below).

Group V: Approaches **A15** – **A20** comprise surface charge modifications irrespective of identifying aIL interaction sites or changes in structural stability due to aIL beforehand. The underlying principle is to repel like-charged solvent molecules by introducing either positively (K/R) or negatively (D/E) charged residues on the protein surface. Introducing charged residues (D, E, R, or K) at all surface residue positions (**A15**), introducing only E there following ref. [17] (**A16**), or substitutions to all other residues but D, E, R, or K (**A20**) led to GiP$_{var}$ = 1.10 to 1.76, but GiP$_{pos}$ < 1.0 with at the same time ≥ 120 residues to consider. Focussing on lysine residues on the surface only and substituting them to E (**A17**) yielded GiP$_{var}$ = 2.26, but GiP$_{pos}$ = 0.33, again indicating that such charge changes are effective at predicted relevant sites with ~1/3 of the precision of random mutagenesis only. Finally, performing positive-to-negative substitutions for surface residues (**A18**) or the opposite, negative-to-positive substitutions (**A19**), yielded almost identical results for both GiP$_{var}$ ≈ 2.75 and GiP$_{pos}$ ≈ 0.56, indicating that the direction of single charge changes does not matter but that such changes are effective at predicted relevant sites with ½ of the precision of random mutagenesis only.

Group VI: Approaches **A21** and **A22** were suggested based on previous observations for the *Bs*LipA SSM library [16] and involved the somewhat unexpected substitutions to chemically different amino acids at all sites, or substitutions in helices and loops. However, in both cases, GiP$_{var}$ and GiP$_{pos}$ are close to 1 or below, indicating that these approaches lead to precisions as

found in random mutagenesis. Not considering this prior information led to overrating the approaches previously [16].

To conclude, of the presented 22 approaches, only two stand out with substantially improved GiP_{var} and GiP_{pos} values. These are **A12**, which exploits experimental information on the thermostability of *Bs*LipA, resulting in GiP_{var} of 3.03 and GiP_{pos} of 1.61, as well as **A14**, which exploits structural weak spots of *Bs*LipA predicted by rigidity theory, resulting in GiP_{var} of 2.39 and GiP_{pos} of 1.41. Further, they only require performing substitutions at 6 to 10 positions. On the other hand, approaches employing the concept of surface charge modification, which focus on repelling aIL ions via the introduction of charged residues at the surface (**A1, A3-A4, A15-A19**), yield high $GiP_{var} \geq 1.6$ (except for **A3**) only at the expense of low $GiP_{pos} \leq 0.6$.
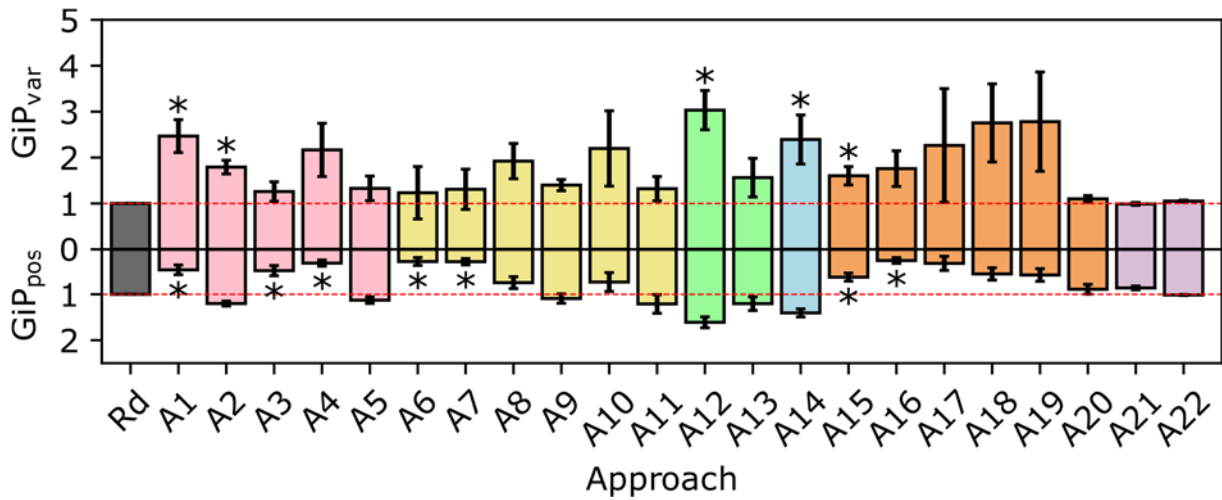
Figure 3: Only five approaches (A1, A2, A12, A14, A15) yield a significantly improved prediction precision for relevant variants compared to random mutagenesis; only two approaches (A12, A14) yield a markedly improved prediction precision for relevant positions compared to random mutagenesis. Approaches are colored according to their classification. See **Figure 2** for the color code. GiP_{var} and GiP_{pos} are shown as mean ± standard error of the mean over the four *Bs*LipA SSM libraries. Significant differences compared to random mutagenesis (**Rd**) are indicated with an asterisk if $p < 0.05$ for each of the four *Bs*LipA SSM libraries.

3.4 Evaluating Physicochemical and Evolutionary Properties for Predicting Improved aIL Resistance

Motivated by recent findings that simple descriptors can explain protein stability change upon substitutions [76], we scrutinized if five physicochemical and evolutionary properties of protein residues can predict relevant variants and substitution sites to improve aIL resistance. These properties are solvent accessibility (**P1-P3**), relative volume (**P4**), hydropathy (**P5**), unfolding free energy (**P6**), and residue conservation (**P7-P8**) (summarized in **Table 1**; see the Supplementary Information and **Table S1** for detailed information on each approach). We also combined these properties (**P9**) and evaluated their robustness towards deviations from the optimal range by relaxing and tightening the ranges by 25% and 50% (**P10-P12**). Here, we considered substitutions to all other amino acids at predicted relevant sites according to the properties **P1-P12**.

P1-P3: Solvent accessibility. Recent studies investigating the thermostability of *Streptococcus sp.* protein G [52] and *Bs*LipA [33] reported increased prediction accuracy when substituting at more solvent-exposed positions compared to buried positions. For increasing aIL resistance, selecting residues with a low to moderate solvent accessibility (SA, **Eq. S5)** had a beneficial effect for $GiP_{var} = 1.46$ and $GiP_{pos} = 1.13$ **(10% < SA ≤ 40%, P1)**. Substituting at solvent-accessible positions (5% ≤ SA), in general, was more favorable ($GiP_{var} = 1.20$ and $GiP_{pos} = 1.07$, **P2)** than at buried (5% > SA) sites ($GiP_{var} = 0.58$ and $GiP_{pos} = 0.85$, **P3)**.

P4: Relative volume. The relative volume (r*V*, **Eq. S7**) reflects that different positions can differentially accommodate volume changes, e.g., substitutions of small, buried amino acids to larger ones are usually disfavorable [52]. Consequently, the precision in predicting improved *Bs*LipA thermostability increased when small-to-large substitutions were excluded [34]. Here, similar to the exclusion of small-to-large mutations, excluding substitutions that markedly increase the occupied volume (r*V* > 1.3) led to $GiP_{var} = 1.09$ but $GiP_{pos} = 0.88$.

P5: Hydropathy. The change in hydropathy (ΔHy, **Eq. S9**) of *Bs*LipA variants is related to the concept of surface charge modification as both aim at modifying polarity, which is widely used to increase aIL resistance [17-20, 49]. Here, the highest GiP were found for a moderate reduction in hydropathy (ΔHy ≤ -4) ($GiP_{var} = 1.21$ and $GiP_{pos} = 0.61$).

P6: Unfolding free energy. The unfolding free energy ($\Delta\Delta G_{unf}$, **Eq. S6**) is an important factor when considering substitutions, as beneficial effects towards aIL resistance must compensate potentially destabilizing effects (higher $\Delta\Delta G_{unf}$) due to substitutions. This concept was previously used to evaluate the cooperativity of *Bs*LipA variants to increase aIL resistance in [BMIM/Cl], where the exclusion of strongly destabilizing variants ($\Delta\Delta G_{unf} \geq 7.52$ kcal mol^{-1}) led to a higher chance of determining cooperative variants [63]. Here, excluding substitutions that moderately destabilized the enzyme ($\Delta\Delta G_{unf} > 4$ kcal mol^{-1}) led to the highest GiP ($GiP_{var} = 1.13$ and $GiP_{pos} = 0.93$).

P7-P8: Residue conservation. Residue conservation (CS) is often analyzed prior to rational mutagenesis approaches to determine residues important for the structure or function of enzymes, such as in the catalytic or ligand binding sites [60, 77, 78]. Reducing the degree of residue conservation below which substitutions are allowed led to an almost linear increase of both GiP_{var} and GiP_{pos}, resulting in $GiP_{var} = 1.22$ and $GiP_{pos} = 1.20$ at CS = 0 (see **P8**). However, as relevant sites and substitutions can coincide with semiconserved positions [33], we used CS ≤ 4 as the limit, which yields $GiP_{var} = 1.13$ and $GiP_{pos} = 1.08$ (**P7**).

P9-P12: Combined properties. We then evaluated the performance when combining the properties **P1, P4, P5, P6,** and **P7**. This yielded $GiP_{var} = 3.35$ and $GiP_{pos} = 1.29$ (**P9**), which are substantially increased GiP values compared to the individual properties. Notably, the result is robust to deviations from the optimal property ranges and still yielded $GiP_{var} \approx 3$ and $GiP_{pos} \approx 1$ when tightening or relaxing the optimal ranges by 25%, respectively (**P10** and **P11**). Finally, relaxing the optimal ranges by 50% (**P12**) yielded $GiP_{var} = 1.85$ and $GiP_{pos} = 0.83$, a performance comparable to that of some experimental approaches (**A1, A2,** and **A4**). Tightening the optimal ranges by 50% led to zero predicted relevant variants and positions. Note that relaxing (tightening) refers to modifying the optimal ranges as to include more (less) variants. I.e., for ΔHy, relaxing (tightening) by 25% means modifying the range from [-∞, -4] to [-∞, -3] (or [-∞, -5]), while the same changes modify the SA-ranges from [0.1, 0.4] to [0.075, 0.5] (or [0.125, 0.3]).

To conclude, the combination of five physicochemical and evolutionary properties (**P9**), which can be computed within a few hours from a static protein structure or sequence information, yielded the, so far, highest GiP_{var} value and the third-highest GiP_{pos} value. At 13 positions predicted to be relevant, substitutions would need to be performed, up to about twice as many as predicted by **A12** and **A14**. The five properties had been optimized individually against the *Bs*LipA SSM library, which may explain the excellent performance of **P9**. Still, if the properties were modified by -25% to +50% (**P10-P12**), $GiP_{var} \geq 1.85$ result, although the GiP_{pos} decreased to ~1 or below.

3.5 Computational Approaches can be Further Enhanced by Combination with Physicochemical and Evolutionary Properties

C1-C9: "Combinations". Finally, we probed if the predictive power of the most promising computational structure- and mechanism-based approaches (**A9**, **A11**, **A14**) can be further improved by combining them with the physicochemical and evolutionary properties (**P9**), which also notably reduces the number of predicted relevant variants and positions, resulting in **C1-C3** (**Table 1**; **Table S1**). Furthermore, we assessed the predictive power when the applied properties deviate by -25% or +25% from the optimal values, resulting in **C4-C6** and **C7-C9**, respectively. In most cases, increases in GiP_{var} result, while GiP_{pos} is sustained (~1) or increased (~1.7). These results indicate that the properties can be used as filters to improve the predictive power for relevant variants and positions. Particularly, the results for **C3**, **C6**, and **C9** indicate that, first, predicting relevant positions by identifying structural weak spots with CNA (**A14**) and, subsequently, filtering the variants and positions using the physicochemical and evolutionary properties (**P9**, **P10**, and **P11**) is a powerful and efficient approach to predict smarter variant libraries at very few positions for improving aIL resistance in protein engineering approaches.

4 Discussion

In this study, we systematically and rigorously evaluated the performance of 22 previously described structure-based approaches to increase aIL resistance. We based our assessment on an experimental *Bs*LipA SSM library, which is, to our knowledge, outstanding with respect to the number and completeness of variants and the variants' screening against four aIL. We show that, surprisingly, most of the approaches yield low GiP values, in particular with respect to predicting relevant positions. Here, 14 approaches perform worse than random mutagenesis ($GiP_{pos} < 1$). Encouragingly, however, exploiting experimental information on the thermostability of *Bs*LipA (**A12**) or structural weak spots of *Bs*LipA predicted by rigidity theory (**A14**) yields GiP_{var} values of 3.03 and 2.39 as well as $GiP_{pos} \approx 1.5$. Furthermore, we demonstrated that the combination of five simple-to-compute physicochemical and evolutionary properties (**P9-P12**) substantially increases the precision of predicting relevant variants and positions of *Bs*LipA for increasing aIL resistance. Finally, we showed that combining these properties with predictions from structural stability analyses of MD trajectories (**C1/C4**) or structural weak spots identified by CNA (**C2, C3, C5, C6,** and **C9**) additionally improves GiP_{var} up to 4-fold to ~10 (**C3**, **C6**, **C9**) and sustains or increases $GiP_{pos} \approx 0.96$-1.77. Furthermore, at most ten relevant positions are predicted, similar to the

number obtained using different random mutagenesis approaches [30, 79-81]. This enables the investigation of substitution combinations for additive or cooperative effects.

Our results are based on the *Bs*LipA SSM library that covers all 181 positions and contains all 3439 variants, each with a single amino acid substitution as confirmed by DNA sequencing [16]. This dataset represents a unique opportunity to evaluate the predictions of approaches to improve aIL resistance, because, in contrast to other biotechnologically relevant enzyme properties such as thermostability and resistance towards detergents and organic solvents, for which databases such as ProTherm [82, 83], ProtaBank [84], and FireProtDB [85] exist, such large-scale data is not available for aIL resistance. Additionally, it is unique in terms of its comprehensiveness and unbiasedness. In comparison, the ProTherm database [82, 83] contains on average ~12 single, ~12 double, and ~1 multiple substitutions for each of the ~1000 proteins stored [86] and is strongly biased towards substitutions to alanine [87]. Thus, outliers in this data may potentially corrupt its evaluation to extract generally applicable rules to improve enzyme properties. Finally, the uniformity of screening conditions applied for the *Bs*LipA SSM library avoids ambiguous results originating from different experimental methods, which was observed for thermostability data of the same variant [88]. Note, though, that enzyme activity determined for the *Bs*LipA SSM library may be influenced by differences in thermodynamic or kinetic protein stability [34, 55] and protein expression [56]. Although in a recent study, these shortcomings were circumvented by reporting comprehensive, domain-wide thermostability data for purified variants of protein G (Gβ1, 56 residues) [52], no such data at large scale is available for aIL resistance.

To evaluate our results, we used rigorous binary classifiers that are not affected by data prevalence and data imbalances and report the results relative to performing random mutagenesis, which accounts for *a priori* probabilities [57, 58]. Subsequently, we determined if the changes of the observed relevant and non-relevant populations were significant using Boschloo's exact test [75]. Five approaches **(A1, A2, A12, A14, A15)** significantly improved GiP_{var} compared to random mutagenesis, but only approaches **A12** and **A14** markedly improved GiP_{pos}, although not significantly (**Figure 3**). The latter is likely due to the small sample sizes evaluated for predicted relevant positions (sometimes a field of the contingency table even contains a zero) [89], although **A12** and **A14** consistently improve GiP_{pos} for all four aIL screened (**A12**: 1.66, 1.46, 1.59, 1.74; **A14**: 1.39, 1.31, 1.52, 1.39). This finding indicates that the *Bs*LipA SSM library may still be too small to allow for a rigorous statistical assessment of approaches that aim at predicting small residue proportions as relevant positions. These limitations will likely become more pronounced when smaller datasets, such as those extracted from the ProTherm database [82, 83] or the Gβ1 dataset [52], are considered.

The prediction precision of approaches that determine relevant positions from experimental structural data (group I), extensive computations (group II), or perform general surface charge modifications (group V), was unexpected considering that in no (group II) or at most 50% of the assessed approaches (groups I and V) GiP_{var} values were > 2, and in no (group V) or at most 40% of the cases GiP_{pos} values were > 1. The low performance needs to be related to the extensive experimental (group I) or computational (group II) work required to predict relevant positions, or the wide use of the approaches (group V) [18-20, 41, 44]. Hence, our assessment demonstrates that approaches should be evaluated on large, unbiased, and complete datasets that allow a thorough analysis of *a priori* information; by contrast, many of the approaches in the three groups have been exemplified based on small numbers of variants or positions only

(e.g., 24 positions in the case of **A1** [90], 23 positions in the case of **A4** [17], or 20-28 positions in the case of **A6-A11** [43]). Notably, predictions of relevant positions in terms of interaction sites or perturbed residues for the *Bs*LipA SSM library based on experimental (**A2**, **A5**) or computational (**A9**, **A11**) work perform almost equally, and only moderately better than performing substitutions at all solvent-exposed positions (**P2**). Furthermore, by most of the approaches of the three groups, many (≥ 20) relevant positions are predicted, which then lead to high numbers of substitutions to be evaluated.

The cases where $GiP_{var} > 2$ but $GiP_{pos} \approx 0.3$ to 0.5 (**A1**, **A4**, **A17-A19**) indicate that charge modifications may be effective but that their effect is strongly position-dependent. This corroborates previous findings that aIL interact specifically with a few surface residues of *Bs*LipA, but also hints at that identifying such interaction sites without evaluating the interaction effect on the protein stability is insufficient [43]. Indeed, when changes in local structural stability originating from such interactions were additionally considered, higher GiP_{pos}, albeit still < 1, are obtained (**A8**, **A10**). Finally, almost identical prediction precisions for R/K substitutions at cationic binding sites (**A6**) versus D/E substitutions at anionic binding sites (**A7**), or K/R→D/E (**A18**) versus D/E→K/R (**A19**) substitutions at solvent-exposed sites, indicate that effects on protein stability due to aIL cations or anions can be equally well counteracted. These results furthermore suggest that cooperative countermeasures may be possible when the respective charge modifications are introduced together [42].

Previously, knowledge gained on a system while improving one property was subsequently used to improve another property [33]. This applies particularly to improving thermostability, which has been described to foster protein evolvability [91, 92] and be related to improvements of resistance to organic solvents [66, 93-96] and detergents [33, 97]. In that respect, knowledge gained for improving resistance to detergents also leads to moderate GiP compared to random mutagenesis for predicting relevant positions and variants for resistance to aIL (**A13**). More remarkably, the largest GiP across all 22 approaches are found if prior knowledge on relevant positions for thermostability is transferred to improving aIL resistance (**A12**), corroborating the relationship between proteins that are stable against temperature and other influence [33, 66, 94-97]. Rather than generating and screening an entire SSM library to perform approach **A12**, knowledge gained during enzyme engineering towards improved thermostability should also be valuable [98-102] if the thermostability screening is more efficient than that for aIL resistance. Finally, many more prediction algorithms have been devised for improving thermostability than aIL resistance, which may also be exploited in this context [103-108]. One such example is CNA, which has been applied in retro- [33, 55, 109-111] and prospective [34, 112, 113] studies to improve protein thermostability previously and has been used before to predict structural weak spots of *Bs*LipA [33, 34, 55]. Applying these weak spot predictions (**A14**) yields the highest GiP_{pos} among all evaluated computational approaches and the fifth-highest GiP_{var} among all evaluated experimental and computational approaches, without the need to tailor the method system-specifically and with only moderate computational costs [33].

We contrasted the performance of the established approaches with that of five physicochemical and evolutionary properties. Such descriptors have been widely analyzed before for improving thermostability [33, 52, 114, 115], but less for aIL resistance [16, 49, 63]. Many of the approaches derived from literature share features with these properties. E.g., substituting to chemically different amino acids [16] (**A21**) is highly similar to introducing moderate changes in ΔHy as this often corresponds to different amino acid types, e.g., aliphatic-to-polar and polar-to-charged [116].

However, our hydropathy-based criterion allows us to exclude substitutions that increase hydropathy, that way limiting changes to increases in polarity, which were suggested to be beneficial for aIL resistance, particularly when introduced at the enzyme surface [16, 49]. Surprisingly, the most noticeable improvements originate from properties that disregard specific knowledge on aIL but originate from general data- or structure-based computations, such as solvent-accessibility, residue conservation, and unfolding free energy. The success of these approaches is likely due to "excluding unbeneficial variants" rather than "predicting beneficial variants", corroborating previous observations for excluding or including specific variants [33, 34, 52, 63, 66].

As all properties that filter on the variant-wise level (**P4-P6**) led to increased GiP$_{var}$ at the expense of decreased GiP$_{pos}$ when applied alone, it is advisable to combine variant-wise descriptors (**P4-P6**) with at least one position-wise descriptor (**P1-P3**, **P7-P8**) to circumvent this drawback. Accordingly, combining such properties (**P9**) not only reduced the numbers of predicted relevant variants and positions to a level realizable by current high-throughput methods [67-73] but also substantially increases the precision of predicting relevant variants and positions of *Bs*LipA for increasing aIL resistance. To probe for the bias introduced in **P9** by optimizing the individual properties against the *Bs*LipA SSM libraries, we assessed the performance of the combination when the properties deviated from the optimal values by -25% (**P10**), +25% (**P11**), and +50% (**P12**). Although the performance of GiP$_{pos}$ dropped to ~1, GiP$_{var}$ remains $\geq$ ~2, which is still higher than that of most other approaches and indicating that the computed ranges are robust against deviations from their optimal values.

Finally, the improved predictive performances of **C1-C9** indicate that structure- and mechanism-based computational predictions can still be markedly improved by applying filters based on physicochemical and evolutionary properties. As another favorable result, few predicted relevant variants and positions were obtained, which allows focussing subsequent experimental efforts. This is important because protein engineering approaches are limited to a few positions if the whole sequence space shall be investigated by substitutions, as the library size increases exponentially [74]. Identified variants can subsequently be employed in additive mutagenesis approaches, such as Computer-Assisted Recombination (CompassR), for creating further improved recombinant variants [46, 63]. For instance, substitutions with $\Delta\Delta G_{unf}$ < 7.5 kcal mol^{-1} were found to be more effectively combinable, indicating that the exclusion of destabilizing substitutions with $\Delta\Delta G_{unf}$ > 4.0 kcal mol^{-1} likely leads to combinable substitutions with synergistic and further improved enzyme resistance to aIL [46, 63].

In contrast to other methods limiting the investigated range of potential substitutions, our approach evaluates substitutions over the whole residue range, filtering on properties that are independent of fixed residue characteristics but instead employing relative property differences. For instance, in surface charge modification approaches including only substitutions to charged residues on the enzyme surface, only ~22% of the beneficial substitutions in the *Bs*LipA SSM library are considered, and many relevant variants are discarded [16]. Furthermore, our approach allows exploiting site-specific measures potentially yielding many relevant variants, such as the introduction of hydrophobic or polar residues, which has rarely been thoroughly investigated [49] compared to surface charge modifications [17-20, 45, 49]. However, previous findings that many of the highest increases in aIL resistance were achieved by introducing hydrophobic or polar residues [49] indicate substantial potential for variants with improved enzyme resistance to aIL based on these substitutions. Thus, our results indicate that a time- and cost-efficient workflow

to improve aIL resistance (**C3**) is given by, first, predicting relevant positions as structural weak spots with CNA (**A14**) and, subsequently, reducing the number of predicted relevant variants there according to the physicochemical and evolutionary properties (**P9**). Notably, this combination is robust against variations of the properties by ±25% (**C6**, **C9**).

In summary, we show for a complete SSM library of *Bs*LipA that the majority of 22 commonly used approaches to increase aIL resistance perform surprisingly poorly compared to random mutagenesis. These findings stress the need to consider *a priori* information and evaluate approaches for improving aIL resistance on large and diverse enough datasets in the future. Notably, however, exploiting experimental information on the thermostability of *Bs*LipA or structural weak spots of *Bs*LipA predicted by rigidity theory stand out favorably with GiP$_{var}$ of 3.03 and 2.39 as well as GiP$_{pos} \approx 1.5$. The combination of five physicochemical and evolutionary properties provides an even more compute-efficient approach with still fair GiP$_{var}$. Finally, combining structural weak spot prediction by rigidity theory (CNA) with the physicochemical and evolutionary properties yields particularly good GiP$_{var}$ = 7.18-9.76 and GiP$_{pos}$ = 1.77. Hence, compared to an unbiased random mutagenesis study, the experimental effort to identify 10 relevant variants will be reduced from screening ~117 randomly selected variants to only ~12 rationally selected variants using approach **C6**. Although these results were obtained for the case of *Bs*LipA, CNA was not system-specifically adapted, and the robustness of the physicochemical and evolutionary properties as to pronounced deviations from their cutoff values was demonstrated. These findings suggest that this combination should be applicable to other enzyme systems for guiding protein engineering approaches towards improved aIL resistance for the use in green industrial approaches.

Table 1: Predictive performance of selected approaches and physicochemical and evolutionary properties to predict relevant variants and positions[a]

Secondary structure of *Bs*LipA

β1 α1 α2/β2 α3 β3 α4 β4 α5 β5 α6 β6 α7

#[b]	Conditions	N_{var}[c]	N_{pos}[d]	GiP_{var}	GiP_{pos}	*p*-value$_{var}$	*p*-value$_{pos}$
Rd[e]	Random mutagenesis [16]	206-462/3439	91-124/181	9%[f]	57%[g]	n.d.[h]	n.d.[h]
A1	Binding sites (X-Ray/MD) → R/K [90]	6-13/44	4-9/24	2.47	0.47	0.01-0.05	≤0.04
A11	Structural stability (CNA) [43]	38-63/437	13-18/23	1.32	1.21	0.01-0.55	0.02-1.00
A12	Thermostability hotspots (Class X) [33]	24-46/114	5-6/6	3.03	1.61	≤0.01	0.03-0.18
A14	CNA weak spots [33]	29-46/190	7-9/10	2.39	1.41	≤0.01	0.10-0.30
A15	5% ≤ SA [i] → D/E/R/K	44-93/466	35-58/123	1.60	0.63	≤0.01	≤0.01
A22	Helix- and loop structures [16]	178-392/2774	75-102/146	1.05	1.01	0.43-0.82	0.80-1.00
P1	10% ≤ SA ≤ 45%	107-216/1254	38-47/66	1.46	1.13	≤0.01	0.05-0.74
P4	0 ≤ r*V* [i] ≤ 1.30	140-316/2170	69-97/157	1.09	0.88	0.23-0.54	0.15-0.27
P5	ΔHy [i] ≤ -4	49-140/731	21-43/87	1.21	0.61	0.01-0.87	≤0.01
P6	ΔΔG_{unf} [i] ≤ 4	189-390/2733	85-119/178	1.13	0.93	0.04-0.35	0.28-0.67
P7	CS [i] ≤ 4	177-394/2641	75-101/139	1.13	1.08	0.08-0.26	0.20-0.64
P9	P1 & P4 & P5 & P6 & P7	20-45/108	9-10/13	3.35	1.29	≤0.01	0.22-0.71
P11	P9 + 25%	34-73/207	14-17/26	2.72	1.01	≤0.01	0.80-1.00
C1	A9 & P9	6-12/35	3-4/4	2.77	1.65	≤0.02	0.08-0.58
C2	A11 & P9	0-6/2-23	0-3/1-3	1.97	1.04	0.01-1.00	0.25-1.00
C3	A14 & P9	4-10/11	1/1	7.18	1.77	≤0.01	n.d.[h]
C4	A9 & P10	1-2/12	1/2	1.17	0.89	0.50-1.00	0.50-1.00
C5	A11 & P10	0-1/5-7	0-1/1	0.84	0.87	0.45-1.00	0.25-1.00
C6	A14 & P10	2-5/5	1/1	9.76	1.77	≤0.03	n.d.[h]
C7	A9 & P11	14-28/77	4-7/10	2.70	0.96	≤0.01	0.50-1.00

C8	A11 & P11	3-11/20-51	1-4/3-6	2.69	1.19	0.01-0.73	0.10-1.00
C9	A14 & P11	13-20/26	2/2	6.87	1.77	≤0.01	n.d.[h]

[a] Substitutions to specific residues are indicated by "→" plus one-letter code; in all other cases, substitutions to all residues are performed. The results for the predicted relevant variants and positions for all evaluated approaches, properties, and the combinations of both are shown along the sequence of *Bs*LipA (see the top for a secondary structure representation): Red bars indicate relevant positions for which relevant variants were correctly predicted. Blue bars indicate relevant positions for which no relevant variant was correctly predicted. The height of red bars represents the fraction of relevant variants among all predicted variants for the given position, thus, describing the precision of predicting relevant variants. The height of blue bars represents the fraction of (falsely) predicted relevant variants of all possible variants at this position, thus, giving an estimate of the experimental work unnecessarily spent when investigating all predicted variants. In all, high red bars and low blue bars indicate a favorable approach, and vice versa. For random mutagenesis (**Rd**), the graph along the *Bs*LipA sequence represents the experimentally determined mutagenesis efficiency (i.e., the relevance) of each sequence position. Thus, blue bars represent positions not relevant in all aIL, whereas red bars represent positions relevant in at least one aIL. The height of red bars displays the average fraction of relevant variants at the respective relevant position.

[b] Numbering of evaluated approaches and properties. **A** = **A**pproach, **P** = **P**roperties, **C** = **C**ombination of approaches and properties.

[c] Number of relevant variants vs. all considered variants.

[d] Number of relevant positions vs. all considered positions.

[e] Random mutagenesis.

[f] Averaged percentage of relevant variants compared to the whole *Bs*LipA SSM library.

[g] Averaged percentage of relevant positions compared to the whole *Bs*LipA SSM library.

[h] Not determined.

[i] See **section 3.4** in the Supplementary Information for an explanation of the abbreviations.

5   Acknowledgments

6 Author contributions

HG designed the study. TEH performed computations. TEH and HG analyzed the data. TEH and HG wrote the manuscript. MDD prepared the dataset and revised the manuscript. KEJ and US revised the manuscript and supervised the creation of the *Bs*LipA SSM library.

7 Declaration of Interest

The authors declare no competing interests.

8 Supporting Information

The Supporting Information includes:

- Supplementary Table S1, Supplementary Figure S1, Supplementary Methods as described in the text, and Supplementary References
- RA_{aIL} values for the complete BsLipA library in four aIL (Excel XLSX file)

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10 Graphical Abstract

Assessing the precision for predicting ionic liquid-resistant enzyme variants

Predictions from literature approaches

Experimentally determined substitution sites

Predictions from a rational approach

Correct prediction
Incorrect prediction