Exploring the full natural diversity of single amino acid exchange reveals that 40–60% of BSLA positions improve organic solvents resistance

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Abstract

Objectives: Protein engineering has been employed to successfully improve organic solvent resistance of enzymes. Exploration of nature's full potential (how many beneficial positions/beneficial substitutions of the target enzyme) to improve organic solvent resistance of enzymes by a systematic study was performed.

Results: We report the results of screening the previously generated BSLA (*Bacillus subtilis* lipase A)-SSM (site saturation mutagenesis) library (covering the full natural diversity of BSLA with one amino acid exchange) in presence of three cosolvents. The potential of single amino acid substitution that nature offers to improve the cosolvent resistance of BSLA was determined by analyzing the number of beneficial positions/substitutions, accessibility and chemical compositions.

Conclusion: Lessons learned from analysis of BSLA-SSM library are: (1) 41–59% of BSLA positions with in total 4–10% of all possible substitutions improve the cosolvent resistance against TFE, DOx, and DMSO; (2) charged substitutions are preferred to improve DOx and TFE resistance whereas polar ones are preferred for DMSO; (3) charged substitutions on the surface predominantly improved resistance while polar ones were preferred in buried "regions". (4) Interestingly, 58–93% of beneficial substitutions led to chemically different amino acids.

Keywords: BSLA, Directed evolution, Protein engineering, Site saturation mutagenesis, Organic solvents resistance

Background

Being able to routinely reengineer enzymes for efficient enzymatic catalysis in organic solvents would be highly attractive for chemical production in general and overcome limitations of biocatalysis in water such as limited solubility of hydrophobic substrates and products, suppression of unwanted side reactions, cost-effective product recovery and thereby avoiding waste water treatment (Arnold 1990; Castro and Knubovets 2003). General principles to redesign and to stabilize enzymes in organic solvents or cosolvents are therefore of high importance and first molecular insights have been discovered (Micaelo and Soares 2007; Li et al. 2010). Enzymes maintain their structural conformations through intramolecular interactions among amino acids and between amino acid and surrounding water molecules. A prerequisite to maintain structural integrity, flexibility and activity in non-aqueous solvents or cosolvent mixtures is that water molecules remain bound to the enzyme to ensure its flexibility (Zaks and Klibanov 1988). Hydrophilic organic solvents (e.g. EtOH, MeOH, and DMSO) with a polarity similar to water are fully miscible (homogeneous solution) (Park et al. 2012) and organic molecules are in close contact with enzymes. The latter can result in a

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competition of solvent molecules with enzyme-bound water molecules and results in structural changes or denaturation (Ogino and Ishikawa 2001; Yang et al. 2004). In contrast, hydrophobic solvents (e.g. heptane, hexane and toluene) exhibit poor solubility in water and compete much less for enzyme-bound water molecules. Therefore, hydrophobic solvents are often unable to remove substantial amounts of water from enzymes (Gorman and Dordick 1992), and often do not affect enzyme activity. In few cases, it was reported that organic solvent molecules can reduce enzymatic activities without altering the enzyme structure for instance by competing with the sixth water ligand of P450 BM3 at the catalytic heme center (Roccatano et al. 2006; Wong et al. 2004).

Enzyme immobilization and supplements of stabilizers (such as salts, sugars, surfactants) have been reported to effectively stabilize enzymes in presence of organic solvents (Stepankova et al. 2013). In the last two decades altering the enzyme stability by protein engineering developed into a powerful alternative to overcome limitations in solvent resistance (Stepankova et al. 2013; Zhao et al. 2015; Markel et al. 2017). Overall, more than 24 reports have been published in which (co-)solvent resistance has been improved (≥ 15 reports by directed evolution; ≥ 9 reports by semi-rational design). Till today, improved organic (co-)solvent resistance has been reported for at least four enzyme classes including monooxygenases, hydrolases, oxidoreductases, and transferases (Wong et al. 2004; Kawata and Ogino 2009, 2010; Koudelakova et al. 2013; Liu et al. 2009, 2013; Martinez and Arnold 1991). The reported beneficial amino acid substitutions provided the insight that how enzymes can be stabilizes organic (co-)solvents. For instance, it was found that substitutions of two charged amino acid residues on the surface of α-lytic protease to hydrophobic residues improved protease stability in presence of hydrophilic solvents (Martinez and Arnold 1991). Interestingly, it was reported that some beneficial substitutions were surface exposed (Park et al. 2012, 2013; Kawata and Ogino 2009; Martinez et al. 1992). Furthermore, the formation of salt bridges and hydrogen bonds led to improved organic solvent stability of the lipase (*Pseudomonas aeruginosa* LST-03) in presence of organic solvents (Kawata and Ogino 2010). However, in the above-mentioned semi-rational design and traditional directed evolution experiments, a limited number of clones was screened (usually a few thousand) and resulted in less than 10 beneficial amino acids substitutions. Due to the limited number of screened variants, a comprehensive picture which includes all possible stabilization modes was not reported.

BSLA belongs to the lipase subfamily I-4, contains 181 amino acids in its mature form and shares 74–80% sequence identity with other members of this subfamily (Arpigny and Jaeger 1999; Bustos-Jaimes et al. 2010). An SSM library covering all natural diversity with a single amino acid exchange (named 'BSLA-SSM library') has been previously reported (Frauenkron-Machedjou et al. 2015; Fulton et al. 2015). The BSLA-SSM library mimics natural evolution, which evolves enzymes by introducing single amino acid exchanges due to its very low mutation frequency (Lynch 2010).

In this report, the potential that nature offers to improve the cosolvent resistance of BSLA was determined by screening the BSLA-SSM library against three cosolvents (TFE, DOx, and DMSO) and statistical analysis of beneficial substitutions. Analysis was performed by determining the number of amino acid positions and substitutions that increase BSLA's organic solvent resistance. Subsequently, amino acid substitution patterns were investigated in respect to accessibility (surface exposed and buried residues), chemical compositions of beneficial substitutions, and their location within structural elements. The latter analysis teaches lessons on how to stabilize BSLA in cosolvents and enables computational-assisted protein engineering for discovering general principles of cosolvent tolerance.

Methods
Chemicals and reagents
All chemicals were of analytical grade or higher and were purchased from Sigma Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), Fluka (Neu-Ulm, Germany), and Carl Roth (Karlsruhe, Germany). Enzymes and dNTPs were obtained from New England Biolabs (Frankfurt, Germany) and Sigma Aldrich (Steinheim, Germany). The HPLC-purified oligonucleotides applied for mutagenesis were obtained from GATC (Konstanz; Germany) and Eurofins MWG Operon (Ebersberg, Germany). The oligonucleotide sequences and growth media are summarized or described in Additional file 1.

Strains and plasmids
The template for all PCRs was the plasmid pET22b(+)-*bsla* WT. For DNA manipulation and recombinant protein expression *E. coli* DH5α and *E. coli* BL21-Gold (DE3) (Agilent Technologies; Santa Clara, USA) were used as hosts for DNA manipulation and recombinant protein expression, respectively. Plasmid purification was performed employing the kit "NucleoSpin Plasmid kit" from Macherey–Nagel (Düren, Germany) according to the supplier's recommendations, and DNA concentrations were quantified using a NanoDrop photometer (NanoDrop Technologies, Germany).

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Site saturation mutagenesis on the whole BSLA gene sequence

SSM (Barettino et al. 1994) libraries targeting the whole *bsla* were generated using degenerated NNS (N = A/T/G/C and S = C/G) oligonucleotide primers encoding all 20 amino acids with 32 distinct codons (Additional file 1: Tables S1, S2) as described in our previous publication (Frauenkron-Machedjou et al. 2015).

Cultivation and expression in 96-well plates

After transformation of the PCR product of SSM libraries, single colonies were grown on LB_{Amp} agar plates. For each SSM library, 92 BSLA clones were picked to 150 μL LB_{Amp} medium in 96-well microtiter plate (MTP) (flat-bottomed, polystyrene plates; Greiner Bio-One GmbH, Frickenhausen, Germany). In addition, three BSLA WT clones and one empty vector clone were used as control in the same MTP. The samples were proceeded with as described previously (Frauenkron-Machedjou et al. 2015).

*p*NPB assay in MTP for BSLA activity measurement

p-Nitrophenyl butyrate (*p*NPB) was used as substrate to detect the activity of BSLA (Shirai et al. 1982). BSLA converts *p*NPB to *p*-nitrophenolate, which can be detected at 410 nm. The activity of the assay was determined by addition of TEA buffer (90 μL, 50 mM, pH 7.4) to supernatant (10 μL) and freshly prepared substrate solution (100 μL) containing *p*NPB (0.5 mM) and acetonitrile [10% (v/v)] in each well. The release of *p*-nitrophenolate was recorded by measuring A_{410} at room temperature over 8 min on a microtiter plate reader (Tecan infinite® M200 Pro Austria GmbH, Männedorf, Switzerland). The standard deviation of the assay was determined by measuring the activity in a 96-well MTP containing only BSLA WT and another 96-well MTP containing only EV. The apparent standard deviation was calculated without subtracting the background; the true standard deviation was obtained after background subtraction.

Optimization of screening conditions in organic solvents

DMSO, DOx and TFE were used in this work to screen the generated SSM-BSLA library. In order to determine the concentration of the three organic solvents for SSM libraries screening, the solvents were supplemented with TEA buffer (p*K*a 7.76, pH 7.4, 50 mM) to obtain organic solvents with different concentrations. All three organic solvents were water-miscible as described in the safety sheets provided by the suppliers Sigma-Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). Besides, the pH of the incubated mixtures (lipase, buffer, organic solvent) as well as the pH of the reaction mixture after product formation was measured. pH shifts of < 0.22 pH units in the presence of organic solvents were observed. Concentrations leading to a residual activity between 30 and 40% of the BSLA WT were chosen to screen the SSM libraries.

Screening of SSM-BSLA library in presence of the three organic solvents

The screening assay with organic solvents was performed in polypropylene MTP (flat-bottomed, polypropylene plates; Greiner Bio-One GmbH, Frickenhausen, Germany), which is resistant to 1,4-dioxane. In each well, supernatant (10 μL) was incubated with the organic solvent solution (90 μL, concentrations in Table 1) or with buffer (control) for 2 h at RT on a microtiter shaker (1000 rpm, Edmund Bühler Microtiter shaker, Hechingen, Germany). Freshly prepared substrate solution (100 μL/well) was added and the A_{410} was measured at RT over 8 min (Tecan infinite® M200 Pro Austria GmbH, Männedorf, Switzerland). For each well, the same amount of supernatant was incubated with or without organic solvent. BSLA resistance (WT or variant) was evaluated as activity in the presence of organic solvent divided by activity in absence of organic solvent (equation in Additional file 1).

For all the analysis, the SSM-BSLA variants are identified by the following symbols: "+" variants with improved resistance ($R_V \geq R_{WT} + 3\sigma$); "=" variants with unchanged resistance ($R_V < R_{WT} + 3\sigma$ and $R_V > R_{WT} - 3\sigma$); "−" variants with decreased resistance ($R_V \leq R_{WT} - 3\sigma$), and "×" variants shows non-detectable activity in buffer under assay condition ($A_V < A_{EV} + 3\sigma$). R abbreviates residual activity (defined in equation 1 in Additional file 1), A: activity, V: variant, WT: wild type, EV: empty vector, and represents standard deviation of the screening system in presence of each organic solvent (see Table 1).

Results

The result section is divided in six parts. The first part deals with the conditions used for the screening of SSM-BSLA library in presence of three organic solvents, and the second part focuses on the analysis of the number of amino acids positions which influence organic cosolvent resistance of BSLA (improved/decreased/unchanged organic solvents resistance and inactivation). In the third part, all substitutions were analyzed to determine the number of single amino acid substitutions that led to increased/unchanged/decreased BSLA resistance towards the three investigated organic cosolvents. In the fourth part, the amino acid substitutions were grouped into four types (aromatic, aliphatic, polar, and charged) and the influence of each type of amino acid on BSLA resistance was depicted. The fifth part focuses on the effect of substituting each type of amino acid with all the

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Table 1 Screening system of BSLA in three organic cosolvents

Organic cosolvent	Conc. (M)[a]	R_{WT} (%)	Standard deviation (%)	
	(% v/v)		Apparent	True
DMSO	8.4 ($\approx$ 60.0)	29.0	8.0	10.5
DOx	2.6 ($\approx$ 22.0)	31.0	6.5	9.6
TFE	2.9 ($\approx$ 12.0)	30.0	7.6	12.0

Conc. concentration, R_{WT} residual activity of BSLA WT. Apparent and true standard deviation values for BSLA WT in the respective organic cosolvents, *DMSO* dimethyl sulfoxide, *DOx* 1,4-dioxane, *TFE* 2,2,2-trifluoroethanol

[a] Concentration used in the incubation step for the BSLA activity assay

four amino acid types and the concluding part summarizes the role that location of amino acid substitutions has on organic solvent resistance of BSLA.

Condition used for screening of SSM-BSLA library in presence of three organic solvents

The SSM-BSLA library is the one previously described (Frauenkron-Machedjou et al. 2015) and contains 3620 possible single variants. All SSM-BSLA variants were screened in presence of three organic cosolvents (DMSO, DOx and TFE). The concentration of the three organic solvents used for the screening system was selected to obtain a residual activity of 30–40% of the BSLA WT (Table 1). True standard deviations of 9.6–12.0% were obtained for the BSLA screening systems with the three organic cosolvents. Screening systems with true coefficient of correlations around 12% were commonly used in directed evolution experiments (Wong et al. 2007).

After screening of the SSM-BSLA library in buffer and in presence of each of the three organic solvents, residual activity of BSLA variants (R_V) and the residual activity of WT (R_{WT}) were calculated. Based on the Gaussian distribution curve (σ is the true coefficient of correlation of the screening system see Table 1), all the variants were grouped into four categories as described previously in this report.

Number of amino acid positions which influence organic solvent resistance of BSLA

Figure 1 depicts the number of amino acid positions of BSLA, which led to improved/unchanged/decreased organic solvent resistance as well as inactivation of BSLA. For the organic solvent resistance type "+", it was found that DMSO (107 positions; 59%) yields 32 more positions than 1,4-dioxane (75; 41%) and 33 positions more than TFE (74; 41%), respectively. For the organic solvent resistance type "=", at each of the 181 amino acid positions at least one amino acid substitution was found that did not influence organic solvent resistance of BSLA. For

the organic solvent resistance type "−", it was found that 1,4-dioxane (136, 75% of all positions) yields more positions than TFE (120; 66%) and DMSO (118; 65%). For the resistance type "×", at least one amino acid substitution representing the wild type amino acid was found at 99 (55%) amino acid positions, which inactivated BSLA already in the buffer solution. Detailed analysis of the number of substitutions at each position of BSLA as well as the overall number of amino acid substitutions on secondary elements of BSLA leading to improved/decreased/un-changed organic solvent resistance and inactivation are given in Additional file 1: Tables S3–S6. In addition, 3D models of BSLA for visualization of the beneficial positions for all three organic solvents are illustrated in Additional file 1: Figures S1–S3.

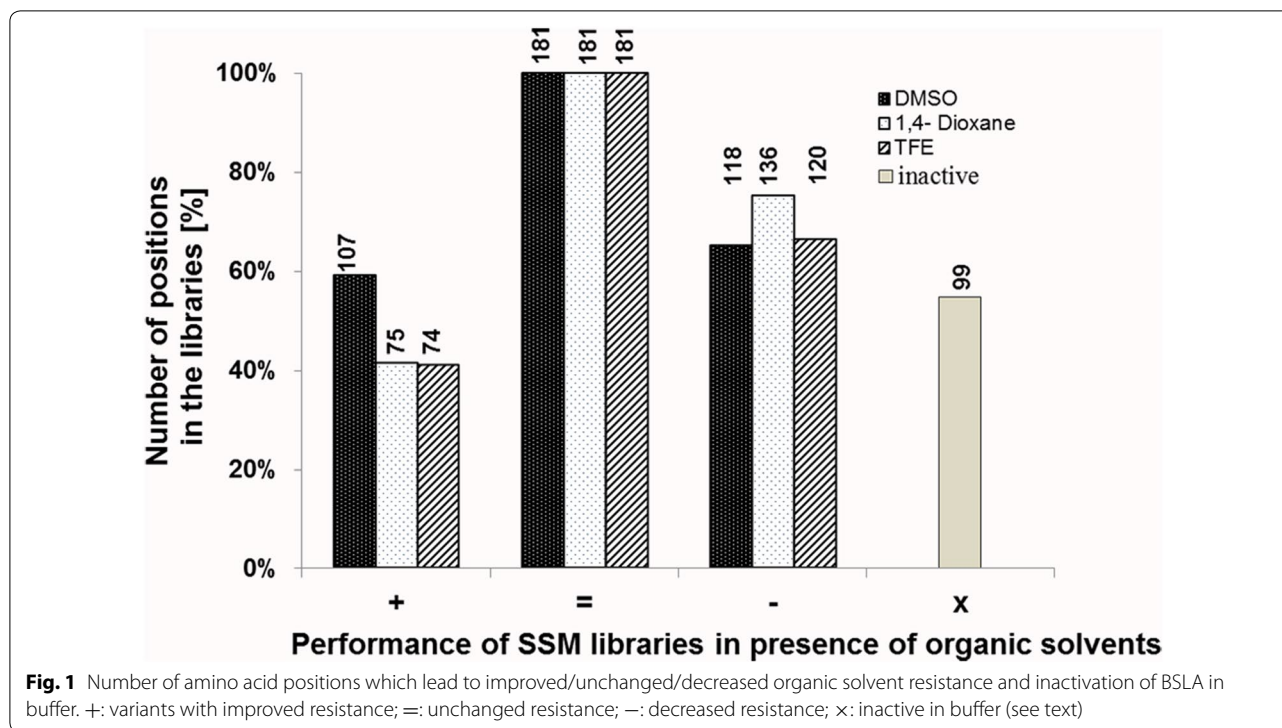### Number of single amino acid substitutions which affect organic solvent resistance of BSLA

Figure 2 shows the number of variants with improved "+", unchanged "=", decreased "−" resistance in presence of DMSO, DOx, and TFE as well as "inactive" variants "×".

For the organic solvent resistance type "+", it was observed that DMSO (371 out of 3620, 10.2% of all substitutions) yields 190 substitutions more than TFE (181, 5.0%) and 212 more than DOx (159, 4.4%). Among the variants with increased resistance towards TFE, 0.17% showed a resistance improvement higher than 2.5-fold, which is 6-fold higher than the values obtained for DMSO and DOx (0.03%). Overall, 61.1–63.2% (2212–2288 out of 3620) of all the substitutions had no effect on BSLA resistance type "=" towards the three selected organic cosolvents. For the organic cosolvent resistance type "−", similar results were achieved in DOx (675 out of 3620, 18.6% of all substitutions) and TFE (664, 18.3%) which are 136 and 125 substitutions more than in DMSO (539, 14.9%). For the type "×", 498 (13.7%) substitutions resulted in inactivation of BSLA.

The influence of amino acid "types" (aromatic, aliphatic, charged, polar) on BSLA's organic solvent resistance

The 20 amino acid substitutions were grouped into four "types" in order to be consistent with previous reports (Wong et al. 2007, 2006; Verma et al. 2014). 15% aromatic (F, Y, and W), 25% aliphatic (A, V, L, I and G), 25% charged (D, E, H, K and R) and 35% polar (termed neutral in the cited papers; C, M, P, S, T, N and Q) amino acids. Cysteine (C), glycine (G), and proline (P) could also be classified as special amino acids. Figure 3 illustrates the effect of each type of amino acid substitution on the resistance of BSLA towards the three organic cosolvents. Inactive variants were defined as variants which showed no detectable activity in buffer.
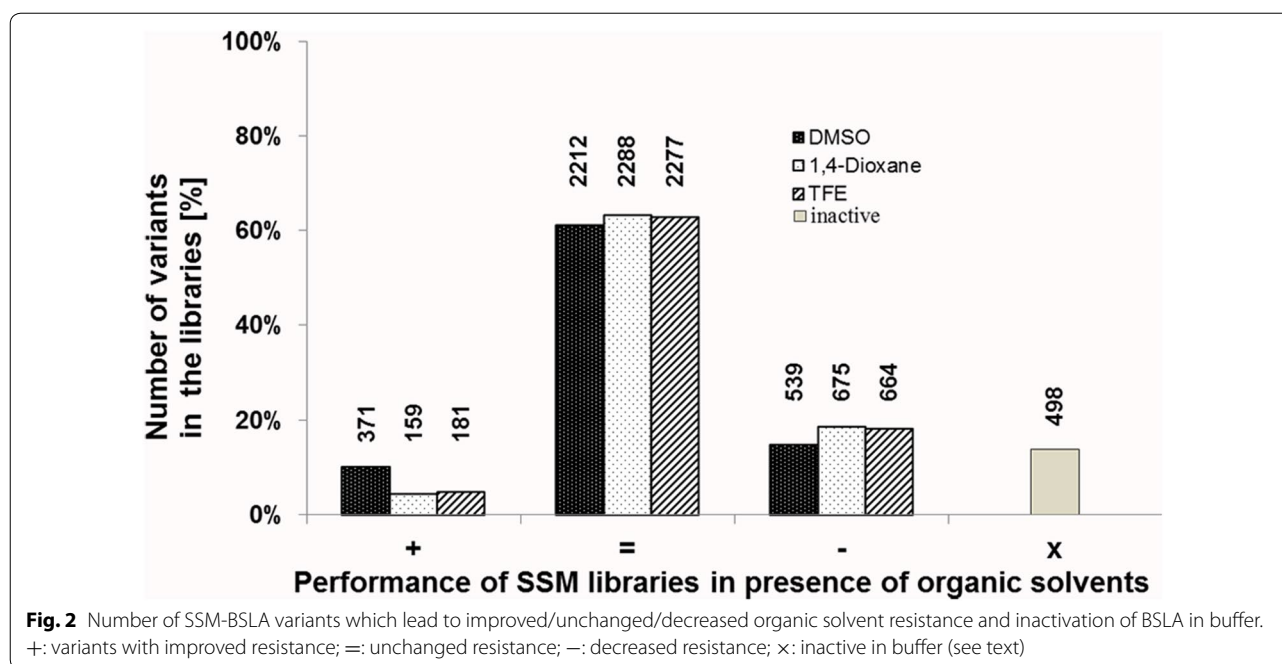
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Fig. 1 Number of amino acid positions which lead to improved/unchanged/decreased organic solvent resistance and inactivation of BSLA in buffer. +: variants with improved resistance; =: unchanged resistance; −: decreased resistance; ×: inactive in buffer (see text)

Supplementing of organic solvent did not in single case lead to regain BSLA activity. In total, 183 out of 498 inactive BSLA variants ("×" type in buffer) harbored charged (37%), 152 polar (31%), 87 aromatic (17%), and 76 aliphatic substitutions (15%).

Figure 3a–c show the number of variants displaying the resistance type "+" towards DMSO; out of 371 beneficial substitutions 126 were to polar (34%), 117 to charged (32%), 88 to aliphatic (24%), and 40 to aromatic amino acids (11%). In case of DOx the 159 beneficial substitutions were composed of 62 to charged (39%), 47 to polar (30%), 26 to aliphatic (16%), and 24 to aromatic amino acids (15%). 181 beneficial substitutions were obtained for TFE. 62 to charged (34%), 57 to polar (31%), 34 to aromatic (19%), and 28 to aliphatic substitutions (15%). Overall, BSLA variants with substitutions to charged amino acids were the majority and therefore preferred type of substitutions to improve organic cosolvents' resistance of BSLA towards TFE and DOx. In case of DMSO, substitutions to polar amino acids were the preferred substitution type. The highest number of improved BSLA variants was obtained for DMSO (126) followed by DOx (62) and TFE (62) (Fig. 3a–c).

For the organic solvent resistance type "=", a similar trend of amino acid substitution was observed for all three organic solvents (36% to polar, 28–29% to aliphatic, 20–22% to charged, and 14% to aromatic substitutions; Additional file 1: Table S7).

The organic solvent resistance type "−" for DMSO is as follows: from 539 beneficial substitutions, 197 were to polar (37%), 127 to charged (24%), 111 to aliphatic (21%), and 104 to aromatic amino acids (19%); for DOx, 242 out of 675 beneficial substitutions were to polar (36%), 191 to charged (28%), 132 to aliphatic (20%), and 110 to aromatic amino acids (16%); for TFE, 230 out of 664 beneficial substitutions were to polar (35%), 181 to charged (27%), 153 to aliphatic (23%), and 100 to aromatic amino acids (15%). Remarkably, BSLA variants with polar amino acid substitutions largely led to a decrease in cosolvent resistance for all three organic solvents; additionally significant differences in the total number of beneficial substitutions were observed (539 substitutions in DMSO vs 675 in DOx).

For DMSO (Fig. 3a), the number of beneficial substitutions are in order polar > charged > aliphatic > aromatic. A different ranking was observed for DOx (Fig. 3b; charged > polar > aliphatic ≥ aromatic) and TFE (Fig. 3c; charged > polar > aromatic > aliphatic). For the variants with organic solvent resistance type "=", the following order was observed: polar > aliphatic > charged > aromatic for all three organic cosolvents. The number of variants for the resistance type "−" followed the order: polar > charged > aliphatic > aromatic in presence of all three organic solvents. In summary, the main type of beneficial substitutions differs from DOx and TFE (charged preferred) to DMSO (polar preferred).

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Fig. 2 Number of SSM-BSLA variants which lead to improved/unchanged/decreased organic solvent resistance and inactivation of BSLA in buffer. +: variants with improved resistance; =: unchanged resistance; −: decreased resistance; ×: inactive in buffer (see text)

Influence of amino acid substitution types on organic solvent resistance of BSLA: comparison between WT and introduced substitutions

Table 2 summarizes, in a matrix for three cosolvents (TFE, DOx, DMSO) and four substitution types (polar, aliphatic, charged and aromatic), the results on the chemical nature of substitutions patterns to which types of amino acid substitutions are more preferential. For all the three selected organic cosolvents, charged and polar substitutions are the preferred substitution types, except for the case of substituting aromatic ones towards DMSO and DOx. Another trend is that mainly the substitutions to chemically different amino acids led to improved organic solvent resistance with significant differences in preference, except for charged amino acid in BSLA WT. The following paragraphs analyze the substitutions patterns (substitutions to) of polar, aliphatic, charged and aromatic amino acids in respect to the three cosolvents.

When substituting the 15 aromatic amino acids in BSLA WT, 44, 19 and 12 beneficial substitutions were obtained for DMSO, DOx and TFE, respectively. Among them, the two most preferred amino acid substitutions are either charged/aliphatic (for DMSO and DOx) or charged/polar for TFE. Substituting the 79 aliphatic amino acids resulted in 155, 60 and 69 beneficial substitutions for DMSO, DOx and TFE, respectively. The two most favorable amino acid substitutions are charged and aliphatic for all the three selected organic cosolvents. Substituting the 54 polar amino acids led to 108, 56 and 62 beneficial substitutions were obtained for DMSO,

DOx and TFE, respectively. The two most preferred amino acid substitutions for all the three selected organic cosolvents are charged and polar. When substituting the 33 charged amino acids, 64, 24 and 38 beneficial substitutions were obtained for DMSO, DOx and TFE, respectively. Among them, the two most preferred amino acid substitutions are also charged and polar for all the three selected organic cosolvents.
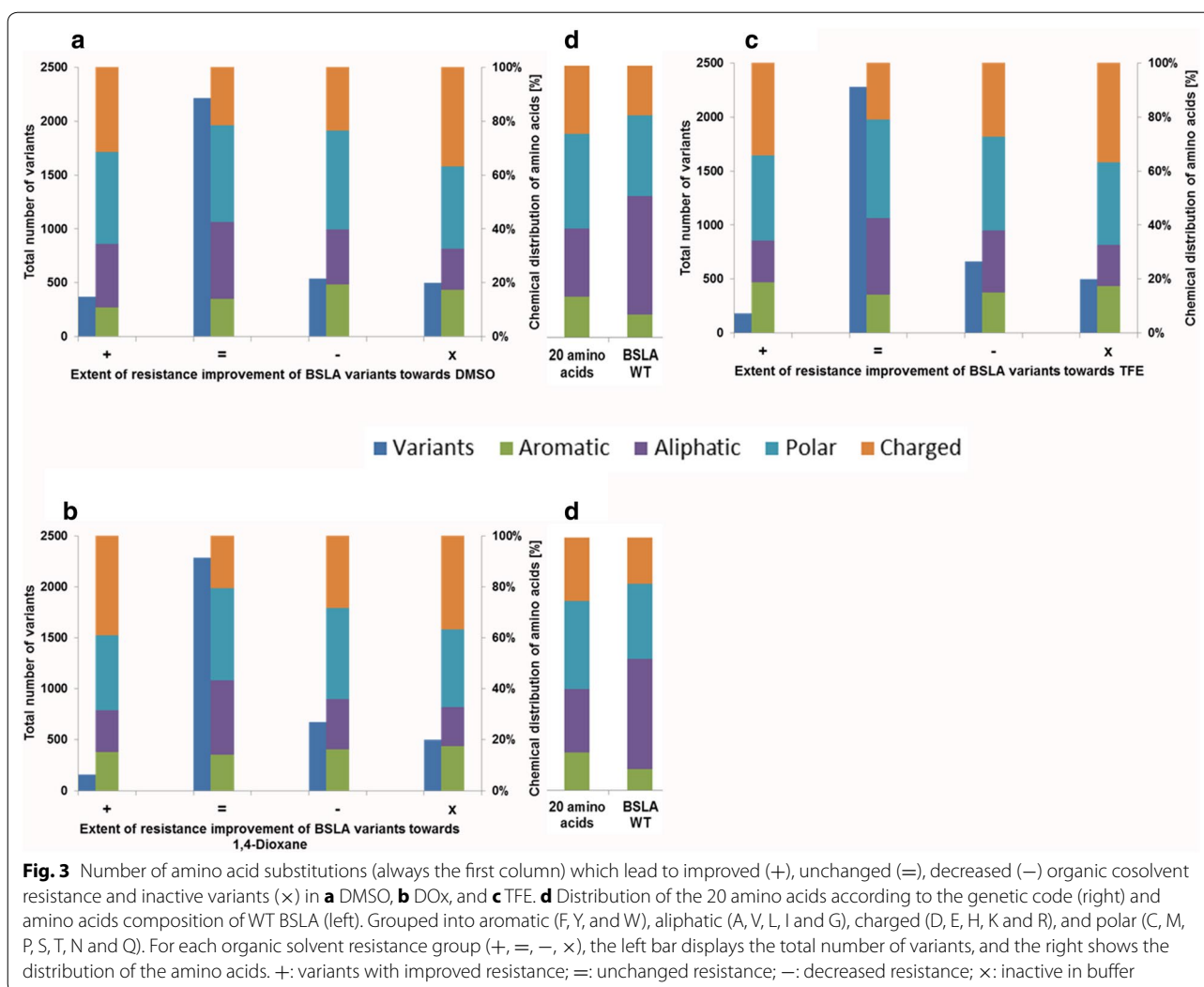
Additional file 1: Tables S8, S9 summarize effects of positively charged and negatively charged amino acids.

Effect of the location of amino acid substitution on organic solvent resistance of BSLA

Around 71% amino acid residues of BSLA are surface exposed and 29% are buried (Table 3). The solvent accessible surface for each residue was calculated using the Accelrys Discover Studio software package employing a water probe with a radius of 1.5 Å and a cutoff of < 5 solvent accessibility for buried residues (Chothia 1976) based on the chain A of the BSLA X-ray crystal structure (PDB ID: 1I6 W) (van Pouderoyen et al. 2001).

Both exposed and buried amino acid positions led to improved organic solvent resistance ("+") for all the four types of substitutions. At exposed positions, more beneficial substitutions were obtained than at buried positions for all the three organic cosolvents. On average, there are 1.1–2.1 beneficial substitutions per exposed position but only 0.4–1.8 beneficial substitutions per buried position.

At exposed positions, the highest number of improved variants (type "+") was obtained with charged

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Fig. 3 Number of amino acid substitutions (always the first column) which lead to improved (+), unchanged (=), decreased (−) organic cosolvent resistance and inactive variants (×) in **a** DMSO, **b** DOx, and **c** TFE. **d** Distribution of the 20 amino acids according to the genetic code (right) and amino acids composition of WT BSLA (left). Grouped into aromatic (F, Y, and W), aliphatic (A, V, L, I and G), charged (D, E, H, K and R), and polar (C, M, P, S, T, N and Q). For each organic solvent resistance group (+, =, −, ×), the left bar displays the total number of variants, and the right shows the distribution of the amino acids. +: variants with improved resistance; =: unchanged resistance; −: decreased resistance; ×: inactive in buffer

substitutions for all three organic solvents (takes up to 34–41% of all the beneficial substitution at exposed positions). At buried positions, polar substitutions are more preferred to improve organic solvent resistance (type "+") for all three organic solvents, taking up to 38–44% of all beneficial substitutions. As previously observed (Frauenkron-Machedjou et al. 2015), the fraction of polar substitutions which resulted in inactive BSLA variants (type "×") is significantly higher at exposed positions (38%) than at buried positions (26%). Notably, charged substitutions at the buried positions resulted in a higher fraction of inactive variants (type "×"; 41%) than the other three types and polar substitutions predominantly reduced activity (type "−"; 32–41% for all three organic solvents) of BSLA. The amino acids distribution of BSLA WT has more aliphatic amino acids (exposed as well as buried; Additional file 1: Table S10).

Discussion

Being able to routinely employ enzymes in organic solvents would offer exciting possibilities in biocatalysis for chemical production. Many enzyme classes (e.g. hydrolases, oxidoreductases, transferases, etc.) have been successfully tailored toward improved cosolvent resistance by using protein engineering methodologies (Stepankova et al. 2013; Carrea and Riva 2000). These studies provided initial clues to reengineer focused regions of enzymes (e.g. substrate access tunnel and substrate binding pocket) and to strengthen interactions between amino acids (e.g. introducing disulfide bridges for improved organic cosolvent tolerance).

None of these studies explored nature's full potential to improve the targeted enzymes. In a first systematic study a complete saturation mutagenesis library of BSLA (181 AAs; SSM-BSLA library) was screened in presence of

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Table 2 Effect of each type of amino acid substitution on organic cosolvent resistance of BSLA

AA substitutions from each type of AA in BSLA WT																
	15 Aromatic AA in BSLA WT (300 substitutions)				79 Aliphatic AA in BSLA WT (1580 substitutions)				54 polar AA in BSLA WT (1080 substitutions)				33 charged AA in BSLA WT (660 substitutions)			
	+ (44)	= (193)	− (36)	× (27)	+ (155)	= (843)	− (269)	× (313)	+ (108)	= (759)	− (136)	× (77)	+ (64)	= (417)	− (98)	× (81)
DMSO																
Aromatic	7	20	8	0	9	14	17	19	8	14	24	19	22	12	21	15
Aliphatic	32	26	17	19	21	32	20	12	23	28	16	16	25	24	30	25
Polar	27	36	33	44	41	36	39	27	32	36	34	29	25	35	36	43
Charged	34	18	42	37	29	18	24	42	36	22	26	36	28	29	13	17
	+ (19)	= (195)	− (59)	× (27)	+ (60)	= (827)	− (380)	× (313)	+ (56)	= (780)	− (167)	× (77)	+ (24)	= (486)	− (69)	× (81)
DOx																
Aromatic	5	22	3	0	17	13	15	19	16	13	22	19	17	14	19	15
Aliphatic	26	26	25	19	13	34	18	12	16	28	19	16	17	26	22	25
Polar	21	35	34	44	32	37	39	27	32	37	32	29	25	35	32	43
Charged	47	17	37	37	38	16	28	42	36	23	27	36	42	25	28	17
	+ (12)	= (211)	− (50)	× (27)	+ (69)	= (836)	− (362)	× (313)	+ (62)	= (751)	− (190)	× (77)	+ (38)	= (479)	− (62)	× (81)
TFE																
Aromatic	8	19	6	0	23	12	17	19	18	15	14	19	16	15	19	15
Aliphatic	0	27	28	19	14	33	20	12	19	26	28	16	16	26	23	25
Polar	33	34	34	44	29	38	37	27	26	38	31	29	45	33	32	43
Charged	58	20	32	37	33	18	26	42	37	22	28	36	24	26	29	17

Twelve patterns per organic solvent are shown. The absolute number of AA substitutions per type is shown in brackets

Amino acid groups: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). For activity group definitions (+, =, −, ×), see text. Values are given as percentages for easier comparison, as BSLA amino acid composition is not evenly distributed (43.6% aliphatic, 29.8% polar, 18.2% charged and 8.3% aromatic AA)

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Table 3 Effect of amino acid location (buried, exposed) on organic solvent resistance of BSLA when replacing each type of amino acid in BSLA WT

	Location of amino acid positions in BSLA (%)							
	Exposed positions (128; 71%)				Buried positions (53; 29%)			
	+ (275)	= (1777)	− (319)	× (189)	+ (96)	= (435)	− (220)	× (309)
DMSO								
Aromatic	10	15	22	15	14	12	16	19
Aliphatic	25	27	21	17	21	36	20	14
Polar	31	36	33	38	44	36	41	26
Charged	35	23	24	30	22	15	23	41
	+ (138)	= (1903)	− (330)	× (189)	+ (21)	= (385)	− (345)	× (309)
DOx								
Aromatic	16	15	17	15	10	12	15	19
Aliphatic	17	27	19	17	14	39	20	14
Polar	26	35	35	38	52	40	37	26
Charged	41	23	28	30	24	9	28	41
	+ (152)	= (1883)	− (336)	× (189)	+ (29)	= (394)	− (328)	× (309)
TFE								
Aromatic	19	15	15	15	17	12	15	19
Aliphatic	16	27	24	17	10	37	22	14
Polar	30	36	32	38	38	40	38	26
Charged	34	23	29	30	34	11	26	41

Sixteen patterns per organic solvent are shown. The absolute number of BSLA variants per type is shown in brackets

Amino acid groups: aromatic (F, Y, and W), aliphatic (A, V, L, I, and G), charged (D, E, H, K, and R), and polar (C, M, P, S, T, N, and Q). For activity group definitions (+, =, −, ×) see text. Values are given as percentages for easier comparison, as BSLA amino acid composition is not evenly distributed (43.6% aliphatic, 29.8% polar, 18.2% charged, 8.3% aromatic). The number of inactive variants (×) was the same for all organic solvents. Column "×" shows that the number of inactive variants resulted from amino acid substitutions. The activity was measured in a buffer plate which was used as reference (absence of organic solvent) for comparison. Therefore, the number of inactive variants was the same for all four organic solvents

three organic solvents (DMSO, DOx, TFE). Thereby the following questions are addressed: (1) how many single positions can contribute to improved organic cosolvent resistance of BSLA; (2) what types of amino acid substitutions influence and are preferential targets to improve the organic solvent resistance of BSLA; and (3) how does the cosolvent accessibility of amino acid side chains (surface exposed and buried) affect the organic solvent resistance of BSLA.

Overall, a surprising high number of amino acid positions (41–59%; Fig. 1) was identified to be beneficial for cosolvent resistance of BSLA (DMSO 59%; DOx 41%; TFE 41%). The latter corresponds to 4.4–10.2% of all 3620 BSLA variants in the SSM-BSLA library and convincingly explains why cosolvent improved variants can be found after screening of only a few thousand clones in a directed evolution campaign; the obtainable sequence space of BSLA is 20181. Significant differences were found among the tolerance towards the three selected cosolvents, for instance despite that more BSLA variants are found with improved DMSO cosolvent resistance, the number of variants with improvements > 2.5-fold was 5.7-fold higher for TFE (0.17%) than for DMSO and

DOx (0.03%). The higher improvement factors in TFE resistance indicate significant differences in interactions between BSLA and the cosolvents TFE and DMSO/DOx. The latter indicate an unequal potential in optimizing BSLA's tolerance against TFE and DMSO/DOx. Furthermore, the percentage of highly beneficial substitutions (> 2.5-fold improvement) is very low and one can expect that these substitutions would rarely be found in directed evolution campaigns.

The amino acid composition of BSLA (43.6% aliphatic, 29.8% polar amino acids, 18.2% charged, and 8.3% aromatic) was optimized by nature for its performance in aqueous solution with a preference for aliphatic amino acids. The investigation of individual effect of each type of substitutions when replacing each type of amino acids in BSLA WT showed that improved variants were obtainable by substitution with all four types of amino acids: charged (32–39%), polar (30–34%), aliphatic (15–28%), and aromatic (11–19%). Obviously charged and polar amino acid substitutions are the predominant beneficial types of substitutions. Both charged and polar amino acids have been reported to contribute to improved organic solvents resistance of enzymes (Park et al. 2012,

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2013; Chen and Arnold 1991; Song and Rhee 2001; Takahashi et al. 2005; Zumarraga et al. 2007), including methanol, ethanol, DMF, 2-propanol, Acetonitrile and DMSO, etc. Postulated reasons were that charged and polar residues could enable the formation of hydrogen bonds between amino acid residues or between surface residues and water molecules which help in maintaining the enzyme structure intact (Park et al. 2012).

Furthermore, substitution of aromatic, polar, charged, and aliphatic amino acid residues with chemically different ones yielded for all three organic solvents more improved variants (68–95%) than substitutions to chemically similar amino acids. Transversion biased random mutagenesis methods are in respect to transition based methods superior in generating chemically different amino acid substitutions. As a results transversion biased methods will in comparison to transition biased methods reduce screening efforts. A similar preference for transversions was reported for the BSLA resistance towards ionic liquids (Frauenkron-Machedjou et al. 2015) when aromatic/polar/aliphatic amino acids are preferentially replaced by chemical different ones.

What influence has the location of the substitution within BSLA? More beneficial substitutions per positions were obtained at surface-exposed positions than at buried positions (DMSO: 275 vs 96; DOx: 138 vs 21; TFE: 152 vs 29). Computational efforts should therefore preferentially target surface-exposed residues since the probability (the number of possible beneficial substitutions is per position) to identify beneficial positions at exposed positions is higher than at buried positions. Substitutions to charged amino acids on the surface of BSLA (DMSO 35%; DOx 41%; TFE 34%) yielded more improved variants than the other three types of substitutions. This finding applies for all three selected organic solvents. The situation is opposite at buried position of BSLA. At buried positions substitutions to polar amino acids are strongly preferred (DMSO 44%; DOx 52%; TFE 38%) over all three types of substitutions and again the trend is consistent for all three selected organic solvents: (Table 3). The top 10 organic solvent resistant BSLA variants are summarized in Additional file 1: Table S11 and their locations on the structure of BSLA are shown in Additional file 1: Figure. S4. In DMSO, 9 of 10 most beneficial substitutions were polar, suggesting the importance of enhanced interactions (e.g., hydrogen bonds) between substituted residues with surrounding amino acids or waters. Similar trend was observed for the top ten beneficial substitutions towards DOx (8 out of 10 were polar), while only five of ten beneficial substitutions in TFE were polar. Substitutions within BSLA were mostly located in loops (DMSO 44%; DOx 47%; TFE 43%) and on helices (DMSO 34%; DOx 36%; TFE 43%) as described in detail

in Additional file 1: Table S6. In summary, loop regions represent a preferred targeting location for improving solvent tolerance against DMSO, DOx, and TFE. A role of substitutions in loop regions to improve cosolvent resistance has previously been reported (Wintrode and Arnold 2001). Substitutions of surface located residues were in many cases reported to contribute to organic solvent resistance of enzymes (Park et al. 2012, 2013; Kawata and Ogino 2009; Martinez and Arnold 1991; Martinez et al. 1992). Ogino et al. (2007) speculated that charged substitutions on the surface prevent organic solvents molecules to diffuse into the protease from *Pseudomonas aeruginosa* PST-01. Yedavalli and Rao (2013) performed site saturation mutagenesis (SSM) of all the amino acid positions in the loops of a BSLA to find DMSO resistant BSLA variants. In contrast to our study, six beneficial positions were identified and recombined, resulting in a final variant 6SR with 8-fold higher catalytic turnover in 60 vol% DMSO. Three of the six mutations increased the surface polarity suggesting that substitutions with more polar amino acids may be helpful to improve the stability of a protein in a water-miscible organic solvent.

Conclusions

A surprisingly high number of amino acid positions contribute to the improvements in organic solvent resistance of BSLA. Substitutions to charged amino acids on the surface and to polar amino acids at buried positions are the main beneficiaries. Substitutions to chemically different amino acids led to significantly more BSLA variants with improved organic cosolvent resistance than substitutions to chemically similar ones. Significant differences were observed in the evolutionary potential in respect to number of beneficial positions and extend of improvements were found among DMSO, DOx and TFE. Follow-up studies have to confirm if the discovered trends can be transferred to the industrially important enzymes' classes.

Additional file

Additional file 1. Supplymentary information.

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