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Lipase in aqueous-polar organic solvents: Activity, structure, and stability

Md. Zahid Kamal,* Poornima Yedavalli, Mandar V. Deshmukh, and Nalam Madhusudhana Rao*

Centre for Cellular and Molecular Biology, Council of Scientific and Industrial Research, Hyderabad, India

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Abstract: Studying alterations in biophysical and biochemical behavior of enzymes in the presence of organic solvents and the underlying cause(s) has important implications in biotechnology. We investigated the effects of aqueous solutions of polar organic solvents on ester hydrolytic activity, structure and stability of a lipase. Relative activity of the lipase monotonically decreased with increasing concentration of acetone, acetonitrile, and DMF but increased at lower concentrations (upto ~20% v/v) of dimethylsulfoxide, isopropanol, and methanol. None of the organic solvents caused any appreciable structural change as evident from circular dichorism and NMR studies, thus do not support any significant role of enzyme denaturation in activity change. Change in 2D [15N, 1H]-HSQC chemical shifts suggested that all the organic solvents preferentially localize to a hydrophobic patch in the active-site vicinity and no chemical shift perturbation was observed for residues present in protein's core. This suggests that activity alteration might be directly linked to change in active site environment only. All organic solvents decreased the apparent binding of substrate to the enzyme (increased K_m); however significantly enhanced the k_{cat}. Melting temperature (T_m) of lipase, measured by circular dichroism and differential scanning calorimetry, altered in all solvents, albeit to a variable extent. Interestingly, although the effect of all organic solvents on various properties on lipase is qualitatively similar, our study suggest that magnitudes of effects do not appear to follow bulk solvent properties like polarity and the solvent effects are apparently dictated by specific and local interactions of solvent molecule(s) with the protein.

Keywords: lipase; polar organic solvents; hydrolytic activity; protein stability; NMR; differential scanning calorimetry

Introduction

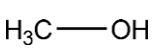
Non-aqueous enzymology explores the potential applicability of enzymes outside their natural conditions and is of immense interest in areas of enzyme biotechnology such as biodiesel and organic synthesis. Numerous industrially important chemical reactions such as peptide and ester synthesis, transesterification, synthesis of pure enantiomers and so forth. were demonstrated to scale up using enzymes as catalysts in non-aqueous solvents.[1–4] Despite well recognized potential uses of enzymes for a wide range of organic reactions, their application is still limited mainly because of their diminished activity in the presence of organic solvents. Excellent reviews on the behavior of enzymes in non-aqueous solvents were presented.[5–8] Water-miscible (polar) organic solvents are found to be far more enzyme deactivating than water-immiscible solvents.[9] It has been shown that enzymes in pure water-immiscible solvents often retain the natively folded structure

Abbreviation: CD, circular dichorism; DMF, dimethylformamide; DSC, differential scanning calorimetry; DMSO, dimethylsulfoxide; PNPB, para-nitrophenyl butyrate

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Md. Zahid Kamal, Nalam Madhusudhana Rao, W112, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad-500007, Andhra Pradesh, India. E-mail: zahid@ccmb.res.in; madhu@ccmb.res.in

Table I. *Physicochemical Properties of Polar Organic Solvents*

Solvent	Structure	Log P[10]	DC[10]	I	E
Acetone		–0.24	78.2	5	21
Acetonitrile		–0.3	64.3	6	36
DMF		–1.01	63.3	6	37
DMSO		–1.35	60.3	7	47
Isopropanol		0.14	70.2	4	18
Methanol		–0.74	30.5	5	32

Log P is the logarithm of partition coefficient in the standard n-octanol/water system. "DC" denotes the denaturation capacity, I denote the polarity index, and E denotes the dielectric constant.

and the activity of enzymes was shown to improve by the addition of very low amount of water and other excipients.[11–13] However, water-miscible solvents have a higher tendency of stripping off tightly bound water from enzyme and ability to partition deeper into the enzyme active site which in turn causes loss of both structure and activity of enzymes.[9,14,15] Nevertheless, experimental proofs for such effects at the level of structure are scarce. It is important to investigate the biochemical and structural properties of enzyme in the presence of polar organic solvents and seek methods to improve structural integrity and activity of enzymes in such solvents.

Lipases are one the most widely used class of enzymes in industries.[2,4,16] They have broad substrate specificity and can catalyze many different reactions, such as hydrolysis and synthesis of esters bonds, alcoholysis, aminolysis, peroxidations, epoxidations, and interesterifications. Such ability to catalyze broad range of reactions also enables lipases finding applications in pharmaceuticals and drugs production, production of biodiesel, food modification and so forth. We have created a thermostable version of a *Bacillus subtilis* lipase using directed evolution. This thermostable mutants named "6B" harbors 12 mutations and showed significant increase in stability, activity, structural rigidity, and reversibility.[17,18] We have solved the crystal structure of 6B lipase and studied the backbone dynamics by solution NMR.[17]

In the present study we investigated the effect of polar organic solvents on activity, structure, and stability of 6B lipase using combination of techniques including circular dichorism (CD), nuclear magnetic resonance (NMR), and Differential Scanning Calorimetry (DSC).

Results and Discussion

Relative activity of 6B lipase in polar organic solvents

Ester hydrolytic activity of 6B lipase was estimated, using para-nitrophenyl butyrate (PNPB) as substrate, in the presence of six polar organic solvents viz. acetone, acetonitrile, dimethylformamide (DMF), dimethylsulfoxide (DMSO), isopropanol, and methanol. Acetone, acetonitrile, DMF, and DMSO are aprotic while methanol and isopropanol are protic in nature. Physico-chemical properties of these organic solvents are listed in Table I. Figure 1 shows the relative activity of 6B lipase in the presence of 0–60% (v/v) of these polar organic solvents. Throughout the text, concentration of organic solvents was given in v/v. Presence of acetone, acetonitrile and DMF decreased the lipase activity in concentration dependent manner [Fig. 1(A)]. Lipase activity is reduced by half in the presence of ~20% of these solvents while 60% of these solvents caused nearly

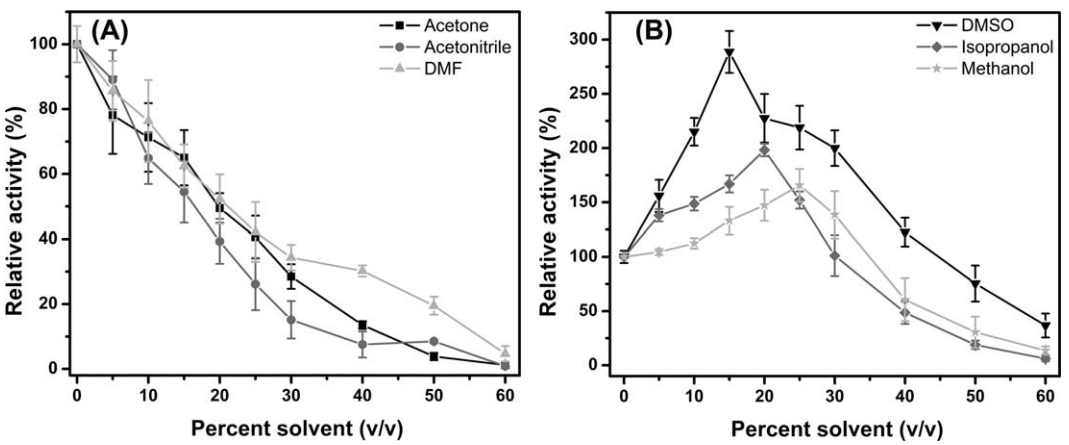


Figure 1. Relative activity of 6B lipase in the presence of organic solvents.

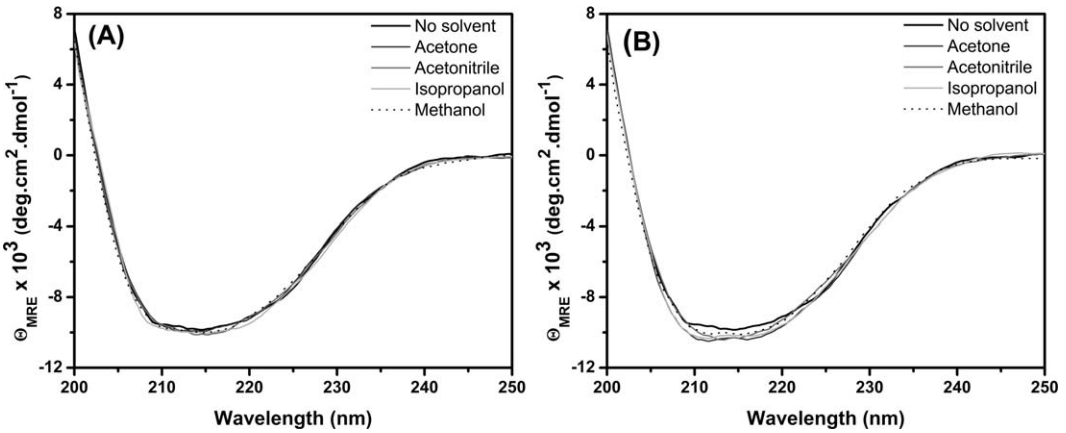


Figure 2. Far UV CD spectra of 6B lipase in the presence of organic solvents. (A) 10% solvents and (B) 60% solvents.

complete loss of activity. Activity profile of lipase is very different in the presence of DMSO, isopropanol, and methanol [Fig. 1(B)]. In the presence of these three solvents, 6B lipase showed an increase in activity at lower concentration followed by a continuous decrease with increase in solvent concentration. Fold enhancement and activity maxima of 6B lipase were ~3 fold in 15% DMSO, ~2 fold in 20% isopropanol, and ~1.7 fold in 25% methanol, respectively. Compared to activity in only buffer, enzyme showed more activity even in ~40% of DMSO and 30% of both isopropanol and methanol. Significant activity (~40%) could be observed even at 60% of DMSO concentration. However, at 60% concentration of isopropanol and methanol activity is close to 10% only. Thus qualitatively different activity profiles of 6B classify these solvents into two distinct sets.

Alteration of enzymes activity in the presence of mixtures of water and solvents is a well documented phenomenon.[6–9] Although enzyme activity change in the mixtures of water and solvents is still poorly understood, numerous factors have been emphasized to bring such effect. Common amongst them are; (i) water or organic solvents might be one of the substrate, (ii) organic solvent molecules might act as specific inhibitor, (iii) the organic solvents changes the bulk properties of solvents such as dielectric constant, polarity and hydrophobicity and so forth, which in turn affect the solvation of substrate and/or transition states and influences their binding to enzyme molecule and (iv) organic solvents changes the structure of enzyme.[19] In the hydrolytic reactions by enzymes such as in the present case, water is one of the substrate. However, in all assays the water volume percent is 40% or more rules out the possibility of water being limiting for the reaction.

In the present study, we saw that activity of 6B lipase decreased in presence of acetone, acetonitrile, and DMF. Nevertheless, activity of 6B lipase showed an increase in the presence of DMSO, isopropanol, and methanol. Such increase in enzyme activity by the presence of organic solvents was seldom observed. For example, Szabó *et al.* observed that peptide hydrolyzing capability of chemically modified papain was increased in the presence of ethanol and acetonitrile; while Tsuzuki *et al.* noticed that presence of polar organic solvents in low concentration increased the ester hydrolytic activity of a lipase from *Rhizomucor miehei*.[20,21] However, mechanistic details of such increase in activity were not investigated.

Structure of 6B lipase in polar organic solvents

We collected far UV CD spectra of 6B lipase in the presence of various organic solvents to probe their effect on the secondary structure of protein. CD spectra of 6B could be collected only in acetone, acetonitrile, methanol, and isopropanol. Presence of DMF and DMSO, which absorb in the far UV spectral region, interfere with the protein signal strongly. Figure 2 shows that secondary structure of 6B lipase remained unperturbed by the presence of all the organic solvents studied. Evidently, observed decrease in activity by the presence of acetone and acetonitrile is not due to any partial denaturation of 6B lipase.

Motivated by the very different effects of solvents on enzyme activity and insignificant effect on lipase structure, we decided to observe changes in the fingerprint amide region in a 2D ^{15}N,^{1}H HSQC in NMR to have a comprehensive understanding of the solvent–protein interactions. Earlier, we have assigned backbone chemical shifts of 6B lipase and could achieve chemical shifts assignments for 150 out of the 181 residues.[17] In the present work, we collected the 2D [^{15}N, ^{1}H]-HSQC spectra of 6B lipase in the presence of 10–40% of various organic solvents. For acetone, acetonitrile, DMSO and methanol, 40% solvent is the highest concentration used, whereas maximum solvent concentrations of 10% and 30% were used for DMF and isopropanol, respectively. Deuterated DMSO and methanol were used for NMR spectra collection while other organic solvents were protonated which limitated studies at

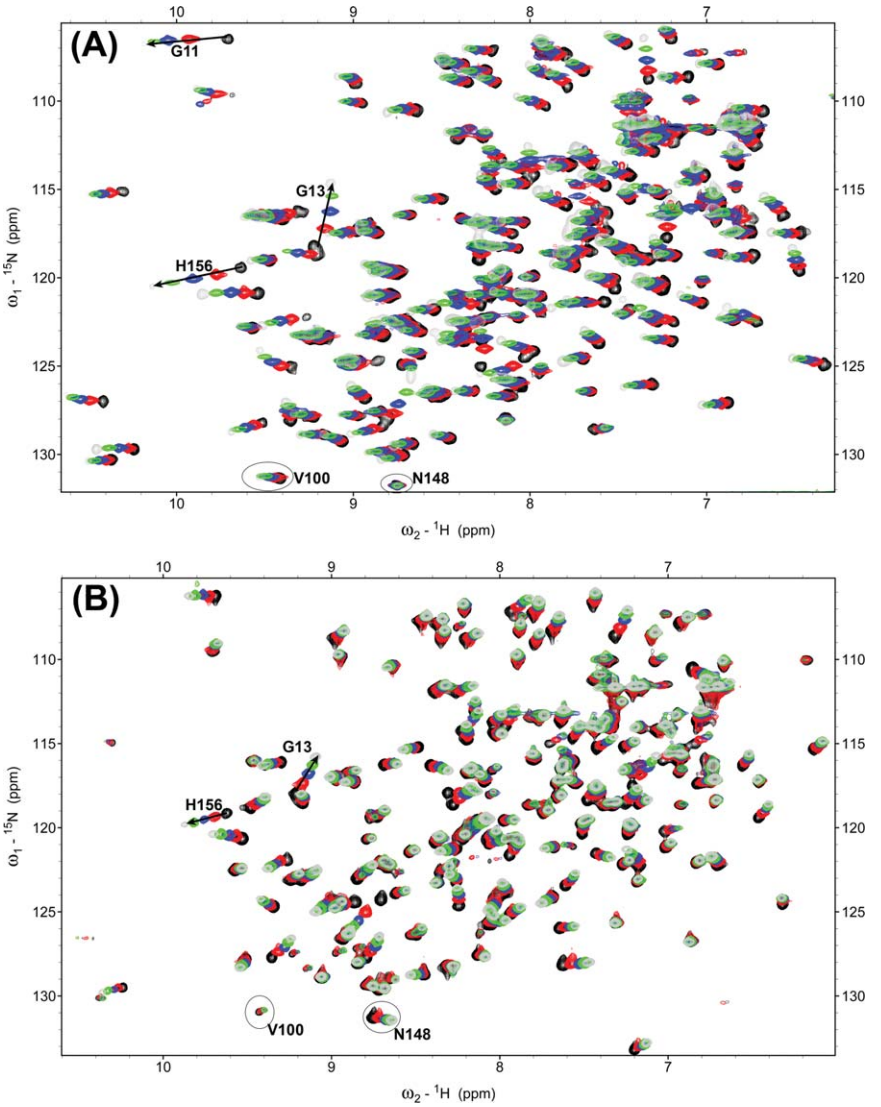


Figure 3. Overlay of 2D [^{15}N, ^{1}H]-HSQC spectra of 6B lipase in the absence and increasing concentration of organic solvents. (A) acetone and (B) DMSO. Examples of significantly perturbed and relatively unperturbed cross peaks are shown by arrows and circles, respectively. No solvent (black), 10% (red), 20% (blue), 30% (green), and 40% (grey). Spectra in the presence of other solvents have been provided in Supporting Information Figures S1 and S2.

higher concentrations of DMF and isopropanol. An overlay of ^{15}N,^{1}H HSQC spectra of 6B lipase in the absence and presence of various organic solvents (Fig. 3) revealed that most of the amide backbone resonances were superimposable suggesting that 6B lipase maintained its native conformation even in the presence of various solvents at all concentrations studied. The observation independently supports findings by CD spectroscopy and established that 6B lipase is indeed highly resistant to structural loss due to the presence of any of the organic solvents studied at concentrations used in this study.

Organic solvents binding sites on 6B lipase

Further, we measured perturbations in ^{15}N,^{1}H HSQC resonances for all the assigned residues. We observe that several residues experience chemical shift perturbations upon titration (Figs. 3 and 4). We also noted that a fewer residues experienced significant changes in the chemical shifts, perhaps due to changes in the hydration shell or due to specific binding of organic solvent molecules. Residues, which demonstrated significantly higher perturbation in backbone amide chemical shifts, were identified for each organic solvent at all concentrations and are listed in Table II. Evidently, all the solvents showed binding preference toward same set of residues. G13, G46, V136, and H156 showed change in the chemical shifts in the presence of all the solvents studied while K44 and L108 had shown change in presence of five of the six solvents barring DMF and acetonitrile, respectively. Other residues such as G11, N48, Y49, V62, Y89, T109, and G153 also showed perturbation in the chemical shifts in the

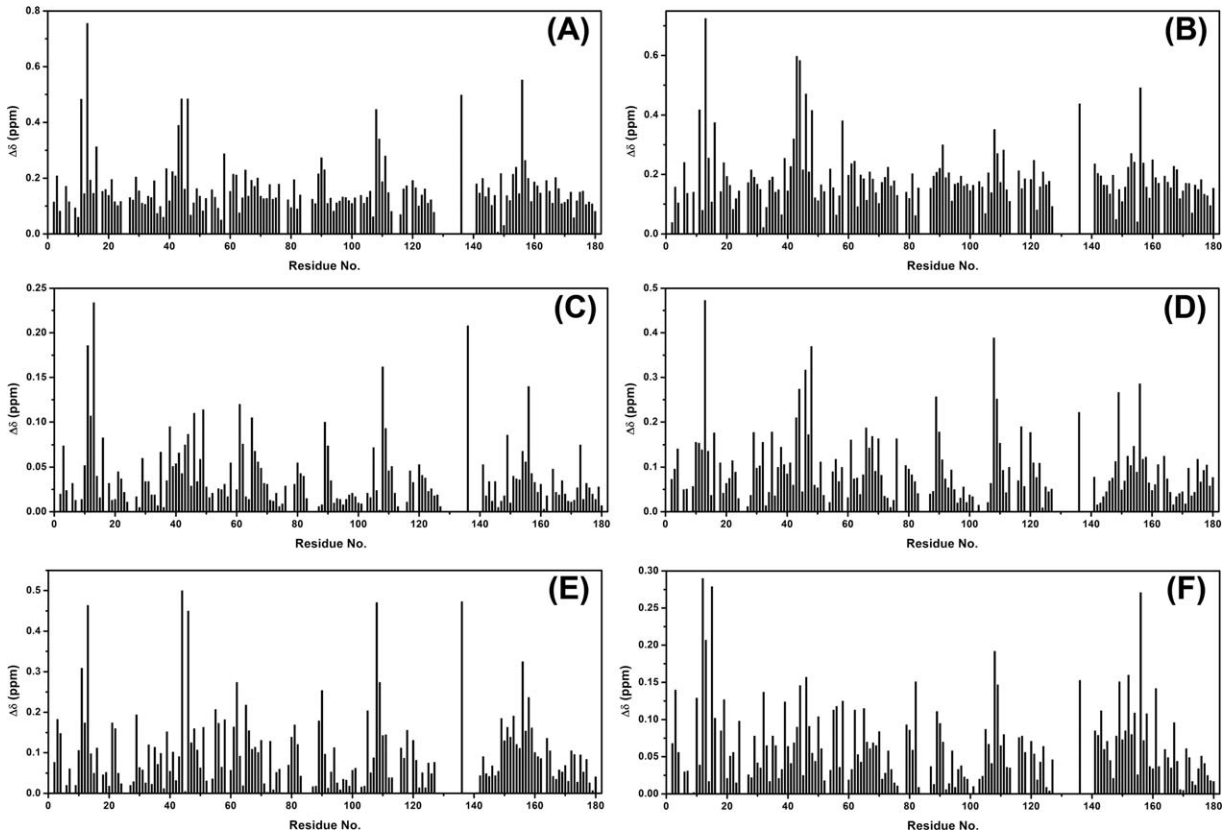


Figure 4. Chemical shift perturbations in the presence of organic solvents as a function of residue number in 6B (A) acetone (B) acetonitrile (C) DMF (D) DMSO (E) isopropanol, and (F) methanol. Data presented here are for 40% of acetone, acetonitrile, DMSO, and methanol; 10% of DMF and 30% of isopropanol. Change in chemical shifts in the presence of other concentrations of organic solvents has been provided in Supporting Information Figures S3–S7). Δδ is the averaged chemical shift perturbation in both nitrogen and proton dimension and is calculated as $\Delta\delta = \mathrm{sqrt}\{(\Delta N/5)^2+(\Delta H)^2\}$.

presence of more than one solvent. Figure 5 shows a surface plot as a function of the amplitude of the perturbation on the three dimensional structure of 6B lipase. As evident from the data, most of these residues are part of same large hydrophobic regions on the protein surface. This is not unexpected considering the organic nature of all the solvents studied. We did not observe any kind of organic solvent penetration to the protein interior. It is also interesting to notice that although magnitude of change in chemical shifts of these residues increased upon increase in the concentration of organic co-solvents, identity of residues remained same for each solvent (Supporting Information Figs. S3–S7). This suggests that even at low concentration of solvents, 6B lipase showed good affinity toward organic molecules and accommodated them at the hydrophobic regions of protein surface. However, protein core of 6B lipase did not show any interaction with solvent even at high concentration and thus may have resisted the possibility of any structure loss.

Other important finding of the NMR experiments is that a significant fraction of these solvent perturbed residues are in the active site or in its close vicinity (Fig. 5). Pouderoyen *et al.* first solved the crystal structure of wild type *B. subtilis* lipase and identified the active site residues (Table II).[22] Essential catalytic component of lipase active site is the "catalytic triad" formed by residues S77, D133 and H156 and "oxyanion hole" formed by peptidic

Table II. *Residues whose Chemical Shifts Undergone Perturbations in Presence of Organic Solvents*

Solvents	Residues faced perturbation in chemical shift
Acetone	G11, G13, K44, G46, L108, V136, H156
Acetonitrile	G11, G13, D43, K44, G46, N48, F58, L108, V136, G153, H156
DMF	G11, G13, G46, Y49, K61, L63, Y89, L108, V136, H156,
DMSO	G13, K44, G46, N48, Y49, E65, Y89, L108, T109, V136, H156
Isopropanol	G11, G13, K44, G46, V62, A105, L108, V136, G153, H156, G158
Methanol	I12, G13, S15, K44, G46, V62, N82, L108, T109, V136, V149, H152, H156, Y161
Active site residues[a]	I12, A15, F17, N18, S77, M78, A105, L108, D133, M134, I135, L140, G155, H156, I157, L160, and Y161

[a] Identity of active site residues is taken from wild type *B. subtilis* lipase.[22]

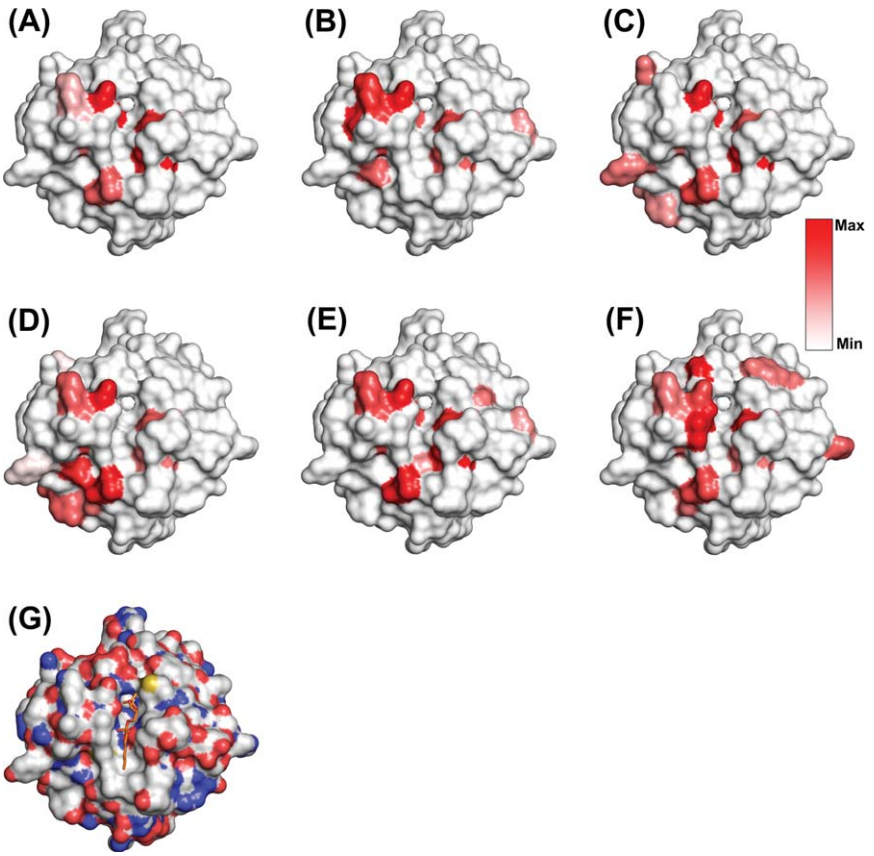


Figure 5. Organic solvents binding sites on 6B lipase molecule. (A) acetone (B) acetonitrile (C) DMF (D) DMSO (E) isopropanol, and (F) methanol. Residues with significant chemical shifts perturbation in 40% of acetone, acetonitrile, DMSO, and methanol; 10% of DMF and 30% of isopropanol are shown in gradient red. Intensity of redness is proportional to magnitude of change in chemical shifts. (G) Molecular surface of 6B lipase is shown in elemental color along with a transition-state analog bound to the active site. Transition-state analog was taken from wild type lipase crystal structure (PDB id: 1R4Z)[23] and modeled into active site of 6B lipase. 180° rotated views of (A–F) have been provided in Supporting Information Figure S8.

group of residues I12 and M78. Chemical shift of H156 was perturbed by the presence of all the organic solvents while that of I12 by the presence of methanol. It is worth mentioning that we did not have assignment of other three essential residues (S77, D133, and M78) hence effect of organic solvent on the environment of these residues could not be identified. Other active site residues that have seen significant perturbation by the presence of organic solvent(s) are S15, L108 and Y161. Amongst the other solvent perturbed residues, G11 and G13 are adjacent to I12, T109 is adjacent to L108 and V136 is adjacent to I135 and should be considered in the immediate vicinity of active site residues and in turn are very capable in influencing the behavior of latter.

As mentioned before, water-miscible organic solvents were implicated to strip off waters from hydration shells of proteins and cause structure loss.[9,14,15] However, we did not notice any such effect with lipase. Our results align well with many previous studies which reported high-resolution structures of proteins obtained from crystals soaked in various organic solvents and found the largely intact structures and hydration shells of proteins while solvent molecules mostly resided in active sites, which were largely dominated by hydrophobic nature.[24–28] It is intuitive to ask why no significant perturbation in hydration shells of various proteins including lipase in the present study could be noticed while other studies strongly suggested otherwise. Investigations using NMR, like in the present case, and X-ray crystallography, usually notice only first hydration shells of proteins. It is very likely that although water-striping in presence of organic solvents usually occurs, the first hydration shell may be relatively resistant to such effect.

Our CD and NMR studies suggest that global structure of 6B lipase was unaffected by the presence of various organic solvents. However, these solvents did induce perturbation in the active site environment. Apparently, binding of organic solvent molecules to active site of enzyme was primarily driven by the hydrophobic nature of active site. Because active site is influenced by the presence of all the organic solvents, it is difficult to ask what

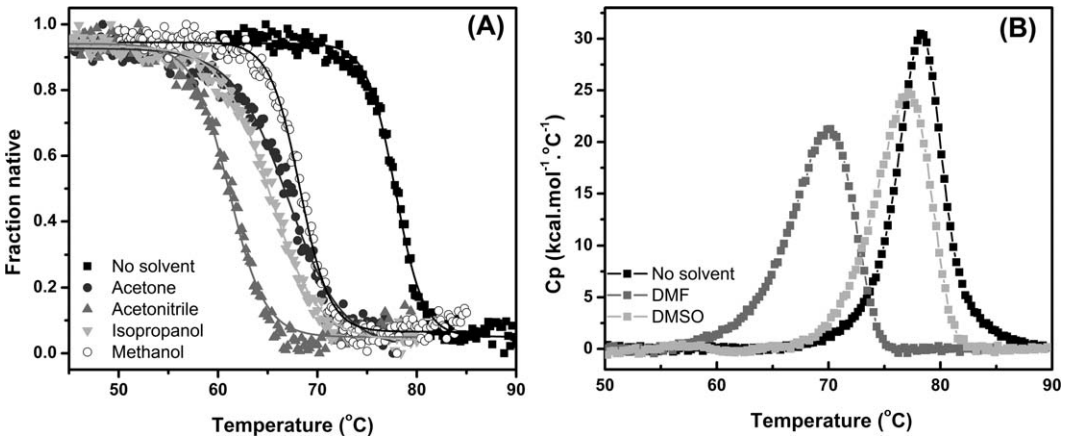


Figure 6. Thermal denaturation of 6B lipase in the presence of organic solvents. Denaturation profiles of 6B lipase in the presence of 20% concentration of organic solvents from experiments performed on (A) CD and (B) DSC.

can be the structural origin of increase in activity in the presence of DMSO, isopropanol, and methanol and how it is different from one in the presence of acetone, acetonitrile, and DMF and also at higher concentrations of DMSO, isopropanol, and methanol.

Stability of 6B lipase in polar organic solvents

Thermal denaturation experiments of 6B lipase in presence of 0–60% of organic solvents were performed using CD spectrometer equipped with Peltier and DSC. As mentioned before acetone, acetonitrile, isopropanol, and methanol did not show interference with CD signal of protein. Thermal denaturation experiments in the presence of these solvents were performed on CD spectrometer at 222 nm wavelength. Figure 6(A) shows the denaturation profile of 6B lipase obtained in these solvents. In the presence of DMF and DMSO, thermal denaturation experiments were performed using differential scanning calorimeter. Figure 6(B) shows the denaturation profile of 6B lipase in the presence of these two solvents. Thermal unfolding experiments could be performed in only 10–20% of DMF and 10–40% of DMSO solutions. Unfolding information at higher concentrations of these solvents could not be obtained because of very high background noise. Thermal unfolding of 6B lipase in buffer only (absence of organic solvents) showed a single transition in both CD and DSC experiments. This unfolding characteristic is maintained during the unfolding of 6B in the presence of all the organic solvents. We determined the melting temperature (T_m) of 6B lipase in the presence of various concentrations of organic solvents. In the unfolding profiles by CD, it is the temperature at which folded fraction is reduced to half while in unfolding profile obtained by DSC it is represented by the temperature at which signal achieved the peak. We also performed unfolding experiments using DSC in 10% of acetone, acetonitrile, isopropanol, and methanol (data not shown) and found that T_m determined from these experiments are in excellent agreement with that obtained from CD. However, experiments at higher concentrations could not be performed because of very high noise in DSC.

As shown in Figure 7, presence of various organic solvents barring DMSO decreased the T_m of 6B lipase at all concentrations studied, suggesting lower conformation stability of lipase in the presence of organic solvents. However, the magnitude of destabilization as suggested by decrease in T_m varied amongst solvents. As apparent from figure, acetonitrile is most destabilizing while DMSO is least. In the presence of DMSO, although a drop of ~2°C in T_m was noticed at 10% concentration, T_m started increasing with further increase in DMSO concentration surpassing that of in the absence of solvent. T_m of 6B lipase was ~78.7 and 80.6°C at 30% and 40% DMSO concentration, respectively, which are higher than T_m in the absence of solvent (~78°C).

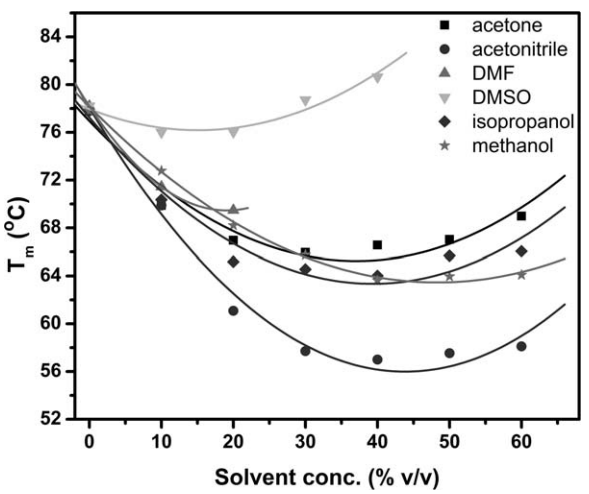


Figure 7. Dependence of melting temperature (T_m) on organic solvent concentration. Trend lines, created using 2nd order polynomial, are provided here to guide the eyes.

For all the organic solvents, decrease in T_m as a function of increasing organic solvent concentration was not linear but it followed an inverse bell-shaped curve attaining a minimum value at particular solvent concentration while increasing at both lower and higher concentrations of organic solvents. The experimental T_m minima attained in various solvents are 65.9°C in 30% acetone, 57.0°C in 40% acetonitrile, 69.5°C in 20% DMF, 76.0°C in 10% DMSO, 64.0°C in 40% isopropanol and 63.6°C in 40% methanol.

It is known that presence of polar organic solvents have tendency to strip off the essential waters from protein molecules that might be important for their structural integrity.[9] Additionally, hydrophobic interaction that is considered primarily responsible for natively folded structure of proteins in aqueous solution would be compromised in the presence of organic solvents.[28,29] Combination of such factors causes a decrease in protein stability that can even lead to its denaturation. Accordingly, T_m should monotonically decrease with increasing concentration of organic co-solvents. Inverse bell-shaped dependence of T_m in the present study is very counterintuitive. This interesting behavior can be understood if simultaneous existence of two effects is considered. On one hand, increasing concentration of organic co-solvents decreases the conformational stability of protein. On the other hand, it decreases the conformational mobility of protein,[30–33] which in turn makes protein less efficient in attaining thermodynamically feasible structure. In effect, presence of organic solvents makes protein thermodynamically less stable but kinetically more stable which in turn can give rise to inverse bell-shaped dependence of T_m on organic solvent concentrations. It will be apt to mention that T_m in the present case did not necessarily represent thermodynamic T_m but is an operationally defined parameter representing midpoint of thermal transition, which might be influenced by contribution from kinetic stability. We indeed could identify the existence of kinetic stability (Supporting Information Fig. S9) in the presence of organic solvents. Such effects on proteins have been well acknowledged in the presence of water-immiscible organic solvents and had been used to explain why enzymes retain their native structure in pure organic solvents.[5] However, such effect of polar organic solvents on proteins, in such concentration range as used in present study, is relatively less known.[34]

All organic solvents increased the k_{cat} *of 6B lipase*

Our results suggest that all the polar organic solvents brought similar effect on the structure and stability of 6B lipase. It appears that similarity in effect of various organic solvents on properties of 6B lipase is primarily driven by their organic/hydrophobic nature. However, effect on activity is different. While acetone, acetonitrile, and DMF caused a continuous decrease in relative activity of lipase with increasing concentration, at low concentration DMSO, isopropanol, and methanol boosted the enzyme activity. How enzyme can accommodate dissimilar effect on activity while other structural and biophysical properties seem to have similar trend?

The complete catalytic cycle of substrate (ester) hydrolysis by *B. subtilis* lipase is an example of ping-pong enzymatic mechanism consisting of two steps, acylation and deacylation (Fig. 8). The essential functional unit of *B. subtilis* lipase is the catalytic triad, which consists of S77, H156 and D133 and oxyanion hole, constituted by peptidic NH groups of I12 and M78. As shown in Figure 8, during acylation steps residues S77 (acting as nucleophilic attacking group) and alcoholic group of ester substrate (acting as leaving group) participate simultaneously in the reaction. Similarly during deacylation step, attacking nucleophilic water and leaving S77 group participate simultaneously. Tetrahedral intermediates form in both acylation and deacylation steps. Evidently, both acylation and deacylation steps of lipase mediated catalysis can be recognized as bimolecular nucleophilic substitution (S_N2) reactions.

Effect of solvent polarity on the rate of S_N2 chemical reactions (non-enzymatic) has been previously investigated. It has been proposed that solvent can influence rate of S_N2 reactions through affecting both nucleophile and reactant (like ester in present case). During the S_N2 reactions, attacking nucleophile assumes an anionic nature. Hence, the factors that can decrease the anionic nature of nucleophile will reduce its nucleophilicity and hence rate of reaction. It has also been proposed that solvents can screen the Coulomb interactions between separated (partial) charges in reactants with the magnitude being proportional to solvent's dielectric constant.[35] Because such Coulomb interactions have significant energetic contributions in increasing rate of the S_N2 reactions, decreasing solvent polarity can increase reaction rate through influencing such Coulomb interactions. So, a more polar solvent will more effectively screen such Coulomb interactions in reactant. Water is a very polar solvent that can form hydrogen bonds with anionic form of nucleophiles and also decrease the Coulomb interactions in the reactant. Hence, replacement of water with organic solvent(s) can increase rate of the S_N2 reaction. Putting the concept in the context of enzymes such as lipase following S_N2 reaction mechanisms for catalysis suggest that reaction speed of such enzymes should increase by decreasing solvent polarity as in the presence of organic solvents. It is apt to mention that chemically modified papain and *Rhizomucor*

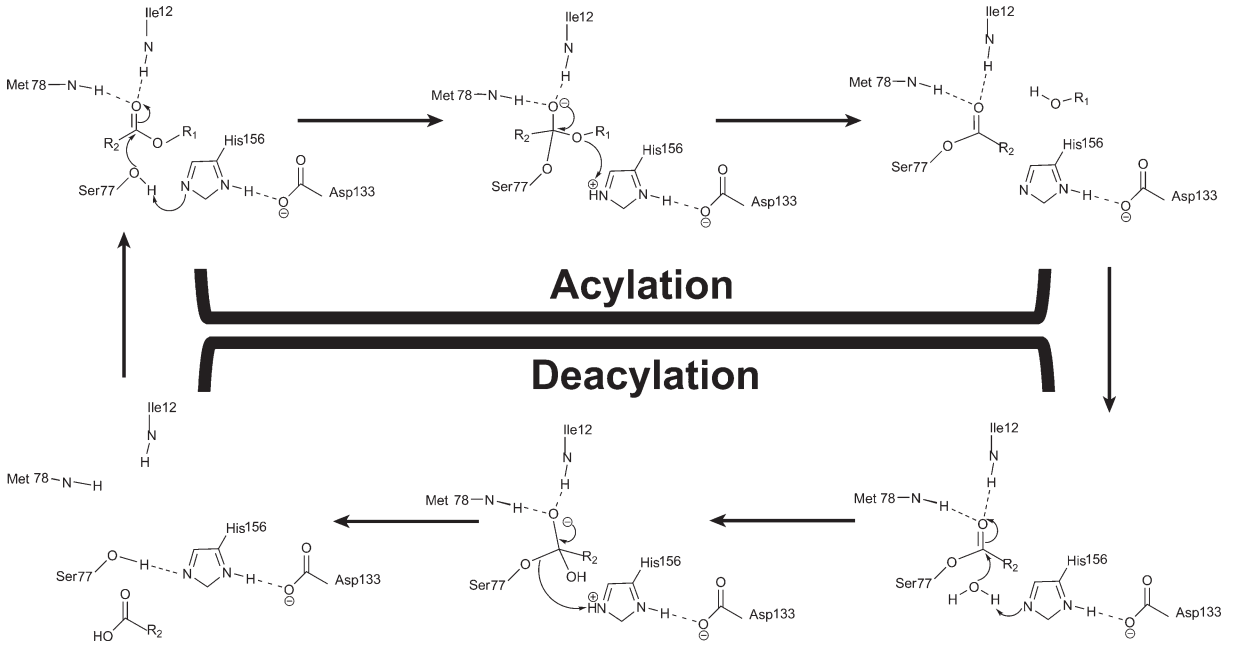


Figure 8. Catalytic mechanism of ester hydrolysis by *B. subtilis* lipase.

meihie lipase whose respective amide and ester hydrolytic activities were reported to be enhanced in aqueous-polar organic solvents also share same S_N2 reaction mechanism.

In the present study, we observed that organic solvent molecules showed binding to the active site (and in the vicinity). So it can be assumed that substrate binding will decrease in the presence of organic solvents. However, once bound, rate of substrate hydrolysis by enzyme should be higher in the presence of organic solvents. Alternatively, in the presence of organic solvents both the enzymatic parameters viz. K_m and k_{cat} should increase. To test such possibility we estimated these enzymatic parameters in the presence of 10% of all the organic solvents. As can be seen in Table III, indeed both K_m and k_{cat} have increased. It should also be noticed that neither K_m nor k_{cat} show any trend following polarity of the organic solvents used, suggesting although the gross effect of solvents is manifesting itself on enzymatic catalytic power, absolute value is influenced by their detail physical and chemical nature and also on the site and nature of interaction of solvent molecules with the protein.

Table III. *Catalytic Parameters of 6B Lipase in the Presence of 10% Organic Solvents*

Solvents	K_m (/mM)	k_{cat} (/min)	k_{cat}/K_m (mM/min)
No solvent	0.19	497	2616
Acetone	2.42	1101	455
Acetonitrile	1.79	630	352
DMF	2.35	1039	442
DMSO	1.08	1441	1334
Isopropanol	0.86	1764	2051
Methanol	3.12	2209	708

Effect of bulk solvent versus molecular physical–chemical properties of polar organic solvents on 6B lipase

Biophysical and biochemical properties of enzymes such as structure, stability, hydration shell, catalytic activity, and substrate specificity are affected by the presence of organic solvents.[5–7] Often such changes in enzyme properties were correlated to the bulk solvent properties such as polarity. For example, water striping ability of organic solvents from enzymes was seen to be dependent on the polarity; higher the solvent polarity, better the ability of the solvent at stripping off water.[14,15] Similarly, extent of reversible/irreversible structural loss and decrease in catalytic activity of enzymes were observed to be related with solvent polarity.[9,36,37] However, the contribution of physical-chemical properties of organic solvent molecules such as structure, shape, size, electrostatics, and hydrogen bonding capability and so forth were not invoked. In the present work, we measured the effect of various polar organic solvents, in a wide range of concentration, on enzymatic activity, structure and stability of a lipase. Our study suggests that effect of all the organic solvents on various lipase properties is following similar trend. In the presence of all the solvents, lipase maintained its structural integrity; its stability showed an inverse bell-shaped dependence on organic solvent concentration; all solvents showed preference in binding to the same site on enzyme (at the hydrophobic patch of active site and immediate vicinity); and enzymatic parameters (K_m and k_{cat}) were boosted by the presence of all the solvents. However, no confident correlation could be drawn between magnitude of solvent effects on proteins

and bulk solvent parameters such as polarity, denaturation capacity and so forth. Neither protein destabilization nor enzymatic parameters showed any relation with organic solvent polarity (Supporting Information Fig. S10). Similarly, although all organic solvents showed binding preference toward the same hydrophobic patch on lipase surface, the exact details of binding residues varies between them (Table II). It appears that although various properties of 6B lipase is "qualitatively" guided by hydrophobic nature of organic co-solvents, hence lower polarity of bulk solvents in comparison to pure aqueous media, the "quantitative" aspects are ruled by physical-chemical nature of organic molecules.

The potential applicability of lipases to boost chemical reactions such as esterification and transesterification with high regio- and enantio-selectivity in organic solvents makes them a very important class of industrial biocatalysts.[2,4,16] However like all proteins, natural lipases are evolved to attain native structure and function in aqueous milieu and often become inactive in organic solvents. Mixture of aqueous-polar organic solvents might provide chance for functionally active enzymes, however they are highly denaturing because of their tendency to strip off essential waters from protein's hydration shell.[9,14,15] Lipases that are structurally stable and catalytically active in the presence of organic solvents are very advantageous because of increased solubility of otherwise sparingly water-soluble substrate and products, increased synthesis over hydrolysis reactions and avoidance of many water-dependent side reactions and so forth.

We examined the effect of increasing concentration of polar organic solvents on the ester hydrolytic activity, structure, and stability of a laboratory evolved thermostable lipase in mixed aqueous-organic solutions. Our investigation led to many interesting facts that can be of importance in designing enzymes to function in water miscible organic solvents. 6B lipase maintained its structural integrity even in the presence of very high concentration of organic solvents, while its structural stability is altered by the solvent. Lowest observed T_m was ~57.0°C in 40% acetonitrile, which is ~21°C lower than that in only aqueous buffer but significantly higher than room temperature. Dependence of T_m on all the organic solvent concentration followed an inverse bell-shaped curve, which is rarely observed and suggests that kinetic trapping of otherwise thermodynamically opposed native structure is feasible even in the aqueous solution of polar organic solvents. We observed that interaction of all the organic solvents to lipase is primarily guided by their hydrophobic nature and all solvents showed preferential binding towards same hydrophobic patch, present in the active site vicinity. No penetration of organic solvent molecules in the protein interior was observed. All the organic solvents caused a decrease in lipase affinity toward ester substrate as inferred from increased K_m. At the same time, all solvents caused significant increase in k_{cat}. Such increase in k_{cat} is rarely reported but we believe that it may be much more generic and can be a distinguished property of enzymes sharing catalytic mechanism to lipases. Our study suggests that although the qualitative nature (trend) of effect of organic solvents on various properties of lipase is grossly governed by the organic nature of solvents, the quantitative aspects are dominated by specific and local interactions of the solvent molecules and their physical–chemical nature. For, comparative purposes, we also investigated activity, structure and stability of wild type *B. subtilis* lipase in the presence of polar organic solvents and found the behavior similar to the 6B lipase (Supporting Information Fig. S11).

Materials and Methods

Protein expression, purification, and concentration estimation

6B lipase was over-expressed and purified following reported methodologies[17,38,39] and concentrations was estimated using modified Lowry method.[40]

Activity measurements

Enzyme activity and catalytic parameters of 6B lipase were estimated at room temperature (~25°C) using PNPB as substrate in the presence of various concentrations of polar organic solvents by methods describe before.[34,35]

Circular dichroism (CD)

Far-UV CD spectra were recorded using JASCO J-815 spectropolarimeter fitted with Jasco Peltier-type temperature controller (CDF-426S/15). All spectra reported are the average of four accumulations. Scan speed of 100 nm/minute, response time of 2 s, bandwidth of 2 nm, and data pitch of 0.2 nm was used for measurement. All spectra were corrected for solution baseline by subtracting the respective blank spectra (50 m*M* sodium phosphate buffer pH 7.2 and various concentrations of organic solvents) recorded identically without protein. Mean residual ellipticity was calculated using equation $[\ominus]_{MRE} = (M_r \times \ominus)/(10 \text{ x } l \times c)$. Where M_r (=115) is the mean residue weight, $\ominus$ is the ellipticity in degree, l is the path length in cm and c is the concentration in mg/mL.

NMR

All NMR experiments were performed on Bruker Avance-II 600MHz spectrometer equipped with 5 mm triple resonance cryoprobe at 25°C. 2D [^{15}N, ^{1}H]-HSQC spectra were collected and processed

using Topspin 2.1 (Bruker Biospin) and using SPARKY.[41] ^{15}N and ^{1}H chemical shifts (BMRB accession number 17647) were transferred to the blank spectra. Titration experiments were started using 0.2 m*M* ^{15}N-labelled 6B lipase in 10% D_2O and 50 m*M* sodium acetate buffer (pH 5.7) in absence of any organic solvent and continued with incremental addition (10%) of organic solvents upto 40% (v/v) concentration. 2D [^{15}N, ^{1}H]-HSQC spectra at each solvent concentration were collected for with 1024×512 data points with 1 s recycle delay. Chemical shifts were calibrated with TPS. Peaks in the titrated spectra were assigned by tracing the chemical shift perturbations with respect to the blank spectra. Change in chemical shift ($\Delta\delta$) upon organic solvent addition was estimated using equation $\Delta\delta = \mathrm{sqrt}\{(\Delta N/5)^2 + (\Delta H)^2\}$. Where ΔN and ΔH are change in ^{15}N and ^{1}H chemical shifts, respectively.

Thermal unfolding on CD machine

Thermal unfolding of lipase variants in the presence of various concentrations of organic solvents (acetone, acetonitrile, methanol, and isopropanol,) was monitored by circular dichroism spectroscopy in a JASCO J-815 spectropolarimeter fitted with Jasco Peltier-type temperature controller (CDF-426S/15). The protein concentration used was 0.05 mg/mL in 50 m*M* sodium phosphate buffer (pH 7.2) with path length of 1 cm. Temperature dependent unfolding profiles were obtained by heating protein at a constant rate of 1°C/minute while measuring the change in ellipticity at 222 nm.

Differential scanning calorimetry (DSC)

Thermal unfolding of 6B was performed by DSC using Microcal VPDSC instrument. 0.25 mg/mL protein in 50 m*M* sodium phosphate buffer (pH 7.2) and various concentrations of organic solvents was used. Samples were degassed extensively before the experiment. DSC scans were performed between 25 and 95°C, at a scan rate of 60°C/hour, with pre-scan thermostat of 10 minutes and a filter time of 16 s. Prior to data analysis, solution/solution base lines were determined and subtracted from protein/solution scans, followed by data normalization for protein concentration.

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References

1. Zaks A, Klibanov AM (1985). Enzyme-catalyzed processes in organic solvents. Proc Natl Acad Sci U S A 82: 3192–3196.
2. Chang SW, Shaw JF (2009). Biocatalysis for the production of carbohydrate esters. N Biotechnol 26: 109–116.
3. Lombard C, Saulnier J, Wallach JM (2005). Recent trends in protease-catalyzed peptide synthesis. Protein Pept Lett 12:621–629.
4. Gotor V (2002). Lipases and (R)-oxynitrilases: useful tools in organic synthesis. J Biotechnol 96:35–42.
5. Klibanov AM (2001). Improving enzymes by using them in organic solvents. Nature 409:241–246.
6. Klibanov AM (1997). Why are enzymes less active in organic solvents than in water? Trends Biotechnol 15: 97–101
7. Serdakowski AL, Dordick JS (2008). Enzyme activation for organic solvents made easy. Trends Biotechnol 26: 48–54.
8. Gupta MN (1992). Enzyme function in organic solvents. Eur J Biochem 203:25–32.
9. Wangikar PP, Michels PC, Clark DS, Dordick JS (1997). Structure and function of subtilisin BPN' solubilized in organic solvents. J Am Chem Soc 119:70–76.
10. Khmelnitsky YL, Mozhaev VV, Belova AB, Sergeeva MV, Martinek K (1991). Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis. Eur J Biochem 198: 31–41.
11. Gao XG, Maldonado E, Perez-Montfort R, Garza-Ramos G, de Gomez-Puyou MT, Gomez-Puyou A, Rodriguez-Romero A (1999). Crystal structure of triosephosphate isomerase from Trypanosoma cruzi in hexane. Proc Natl Acad Sci U S A 96:10062–10067.
12. Zaks A, Klibanov AM (1988). The effect of water on enzyme action in organic media. J Biol Chem 263: 8017–8021.
13. Debulis K, Klibanov AM (1993). Dramatic enhancement of enzymatic activity in organic solvents by lyoprotectants. Biotechnol Bioeng 41:566–571.
14. Gorman LA, Dordick JS (1992). Organic solvents strip water off enzymes. Biotechnol Bioeng 39:392–397.
15. Yang L, Dordick JS, Garde S (2004). Hydration of enzyme in nonaqueous media is consistent with solvent dependence of its activity. Biophys J 87:812–821.
16. Rodrigues RC, Fernandez-Lafuente R (2010). Lipase from Rhizomucor miehei as an industrial biocatalyst in chemical process. J Mol Cat B- Enzym 64:1–22.
17. Kamal MZ, Ahmad S, Molugu TR, Vijayalakshmi A, Deshmukh MV, Sankaranarayanan R, Rao NM (2011). In vitro evolved non-aggregating and thermostable lipase: structural and thermodynamic investigation. J Mol Biol 413:726–741.
18. Kamal MZ, Mohammad TA, Krishnamoorthy G, Rao NM (2012). Role of active site rigidity in activity: MD simulation and fluorescence study on a lipase mutant. PLoS One 7:e35188.
19. Mozhaev VV, Khmelnitsky YL, Sergeeva MV, Belova AB, Klyachko NL, Levashov AV, Martinek K (1989). Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures. Alpha-chymotrypsin and laccase in mixed water/alcohol, water/glycol and water/formamide solvents. Eur J Biochem 184:597–602.
20. Szabó A, Kotormán M, Laczkó I, Simon LM (2009). Improved stability and catalytic activity of chemically modified papain in aqueous organic solvents. Process Biochem 44:199–204.

21. Tsuzuki W, Ue A, Nagao A (2003). Polar organic solvent added to an aqueous solution changes hydrolytic property of lipase. Biosci Biotechnol Biochem 67: 1660–1666.
22. Pouderoyen GV, Eggert T, Jaeger KE, Dijkstra BW (2001). The crystal structure of *Bacillus subtilis* lipase: a minimal alpha/beta hydrolase fold enzyme. J Mol Biol 309:215–226.
23. Droge MJ, Boersma YL, van PG, Vrenken TE, Ruggeberg CJ, Reetz MT, Dijkstra BW, Quax WJ (2006). Directed evolution of Bacillus subtilis lipase A by use of enantiomeric phosphonate inhibitors: crystal structures and phage display selection. Chembiochem 7: 149–157.
24. Fitzpatrick PA, Steinmetz AC, Ringe D, Klibanov AM (1993). Enzyme crystal structure in a neat organic solvent. Proc Natl Acad Sci U S A 90:8653–8657.
25. Schmitke JL, Stern LJ, Klibanov AM (1998). Organic solvent binding to crystalline subtilisin1 in mostly aqueous media and in the neat solvents. Biochem Biophys Res Commun 248:273–277.
26. Mattos C, Bellamacina CR, Peisach E, Pereira A, Vitkup D, Petsko GA, Ringe D (2006). Multiple solvent crystal structures: probing binding sites, plasticity and hydration. J Mol Biol 357:1471–1482.
27. Dechene M, Wink G, Smith M, Swartz P, Mattos C (2009). Multiple solvent crystal structures of ribonuclease A: an assessment of the method. Proteins 76:861–881.
28. Herskovits TT, Jaillet H (1969). Structural stability and solvent denaturation of myoglobin. Science 163: 282–285.
29. Herskovits TT, Gadegbeku B, Jaillet H (1970). On the structural stability and solvent denaturation of proteins. I. Denaturation by the alcohols and glycols. J Biol Chem 245:2588–2598.
30. Burke PA, Griffin RG, Klibanov AM (1993). Solid-state nuclear magnetic resonance investigation of solvent dependence of tyrosyl ring motion in an enzyme. Biotechnol Bioeng 42:87–94.
31. Soares CM, Teixeira VH, Baptista AM (2003). Protein structure and dynamics in nonaqueous solvents: insights from molecular dynamics simulation studies. Biophys J 84:1628–1641.
32. Guinn RM, Skerker PS, Kavanaugh P, Clark DS (1991). Activity and flexibility of alcohol dehydrogenase in organic solvents. Biotechnol Bioeng 37:303–308.
33. Affleck R, Haynes CA, Clark DS (1992). Solvent dielectric effects on protein dynamics. Proc Natl Acad Sci U S A 89:5167–5170.
34. Griebenow K, Klibanov AM (1996). On protein denaturation in aqueousâ'organic mixtures but not in pure organic solvents. J Am Chem Soc 118:11695–11700.
35. Kim Y, Cramer CJ, Truhlar DG (2009). Steric effects and solvent effects on SN2 reactions. J Phys Chem A 113:9109–9114.
36. Gupta MN, Batra R, Tyagi R, Sharma A (1997). Polarity index: the guiding solvent parameter for enzyme stability in aqueous-organic cosolvent mixtures. Biotechnol Progr13:284–288.
37. Zaks A, Klibanov AM (1988). Enzymatic catalysis in nonaqueous solvents. J Biol Chem 263:3194–3201.
38. Acharya P, Rajakumara E, Sankaranarayanan R, Rao NM (2004). Structural basis of selection and thermostability of laboratory evolved *Bacillus subtilis* lipase. J Mol Biol 341:1271–1281.
39. Ahmad S, Kamal MZ, Sankaranarayanan R, Rao NM (2008). Thermostable Bacillus subtilis lipases: in vitro evolution and structural insight. J Mol Biol 381: 324–340.
40. Markwell MA, Hass SM, Tolbert NE, Bieber LL (1981). Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol 72:296–303.
41. Goddard TD, Kneller DG (2004). Sparky-3.112. San Francisco: University of California.