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Article

Spectroscopic Kinetic Monitoring and Molecular Dynamics Simulations of Biocatalytic Ester Hydrolysis in Non-Aqueous Solvent

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SUMMARY

The use of enzymes as catalysts is becoming an increasingly important tool in chemical synthesis, given the mild conditions and high chemoselectivity that can be achieved through enzyme-catalyzed reactions. However, a major limitation in the use of enzymatic biocatalysis is the degradation of enzyme structure and activity in non-aqueous media. Lipases are a subclass of esterases found in most, if not all, living organisms that break ester bonds in lipids. In this study, we explored the effects of various concentrations of non-aqueous organic solvents on pancreatic lipase activity and analyzed the relationships between different properties of solvents and the kinetics of enzymatic hydrolysis through spectroscopic monitoring of a synthetic colorimetric substrate, 4-nitrophenyl acetate. The influence of non-aqueous solvent environments on protein stability was further explored with molecular dynamics (MD) simulations on a 1 nanosecond timescale. Our results suggest a general trend of decreasing enzymatic activity with increasing concentrations of non-aqueous solvent; however, lipase activity in low concentrations of methanol, specifically 5% methanol, was 28% higher than lipase activity in water. Lipase activity in methanol also displayed the greatest rate of hydrolysis of the substrate compared to all other non-aqueous solvents. Interestingly, our MD simulations showed that the conformational state and stability of lipase in methanol is similar to that of the enzyme in water. Lipase activity works the best in 5% methanol which can be used in industry for chemical synthesis and enzyme-catalyzed reactions.

INTRODUCTION

Enzymatic biocatalysis has attracted important applications in chemical synthesis due to greater levels of chemoselectivity, increased atom economy, and reduced production of hazardous byproducts produced by common reagents that are found in common reagents in synthesis (1). In particular, biocatalytic cascades—combinations of

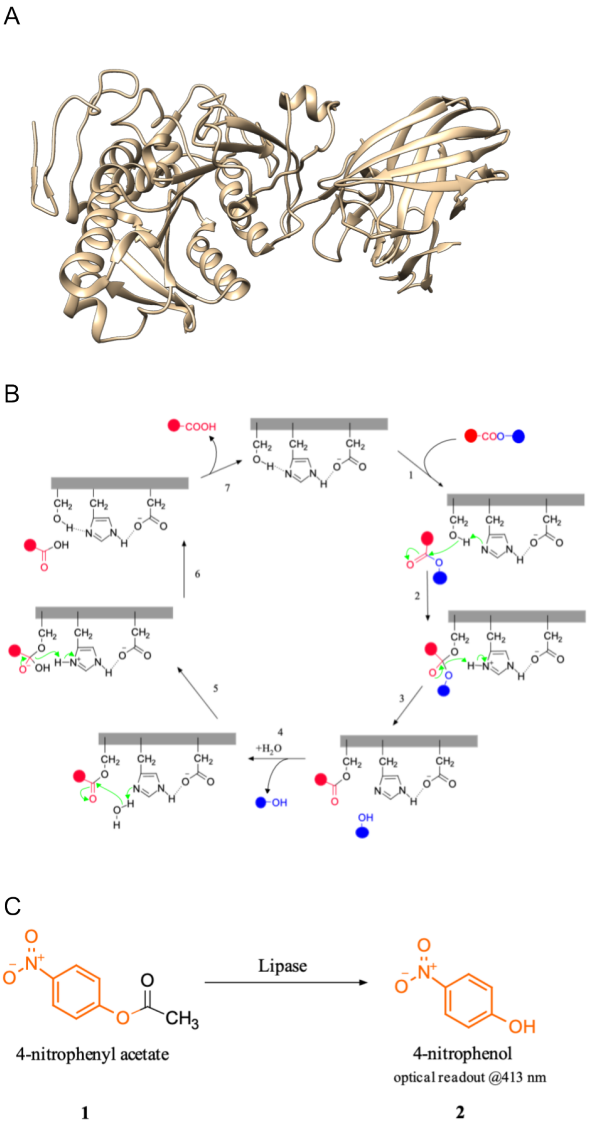

Figure 1. Structure and Mechanism of Lipase. (**A**) Crystal structure of a pancreatic lipase-colipase complex (PDB code: 1N8S). (**B**) Ser-His-Asp catalytic triad coordinated hydrolysis of an ester bond. In the mechanism, the serine nucleophile attacks the carbonyl

carbon of the ester, forming a tetrahedral intermediate, which collapses and releases the alcohol product. Then, a water molecule attacks the carbonyl carbon of the acyl-enzyme intermediate, forming another tetrahedral intermediate, which collapses and releases the carboxylic acid product. Thus, the ester bond has been broken, and the serine nucleophile is free to catalyze another reaction. (**C**) Lipase cleaves the ester bond in 4-nitrophenyl acetate, releasing 4-nitrophenol, which can be tracked spectroscopically at 413 nm. This allows for the quantification of lipase activity.

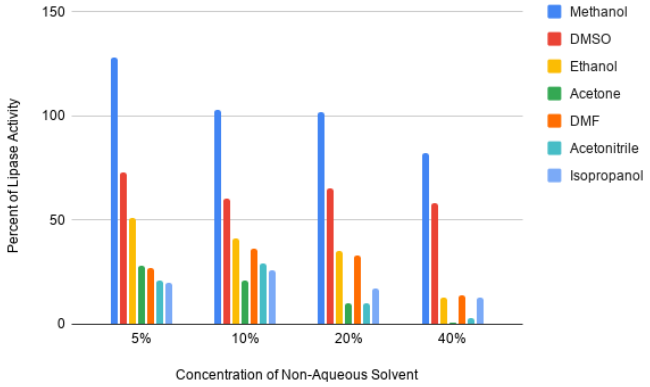

Figure 2. Lipase activity in various concentrations of non-aqueous solvents. Data normalized to percentage of lipase activity in water (100% lipase activity). All the concentrations were prepared in Tris buffer and only one trial was performed.

multiple enzymatic steps—shorten multi-step syntheses by eliminating intermediate steps and reduce hazardous waste (2). Engineered enzymatic biocatalysts have been recently reported in the chemical synthesis of Islatravir (2). *Candida antarctica* lipase B has been reported to catalyze alcoholysis, ammonolysis, and hydrolysis reactions in organic media (3). In addition, biocatalytic processes have been used for the synthesis of chiral intermediates of drugs such as Formoterol and Omapatrilat (4). Deacetylation of phenolic esters can be conducted under acidic or basic conditions, but the utilization of enzymatic catalysis allows the same reaction to be conducted under neutral pH conditions (5).

Lipases—a subclass of esterases—are enzymes that catalyze the hydrolysis of ester bonds in lipids (**Figure 1A**) (6). In addition, lipases have been previously reported to catalyze esterification, interesterification, and transesterification reactions in non-aqueous media (7). For this reason, lipases are used as industrial biocatalysts in the food, detergent, and pharmaceutical sectors, and they also have potential applications in the leather, textile, cosmetic, and paper industries. Given the potential utility of lipase biocatalysis, understanding the effect of different solvent systems on lipase activity is largely applicable.

The digestion of fats in the stomach is catalyzed by three enzymes, one of which being pregastric lipase which originates in the glossoepiglottic area. The active site of pre-gastric lipase consists of a serine nucleophile (Ser152), which is one of the three amino acid residues that make up the catalytic triad (**Figure 1B**), the other two being His263 and Asp176 (8). These residues form a charge-transfer relay network which serves to increase the nucleophilicity of Ser152. This allows the serine nucleophile to cleave an ester bond.

Understanding the effect that different solvents have on the retention of catalytic activity in enzymes is critical in informing the choice of reaction conditions for conducting biocatalytic chemical reactions. The effects of both aqueous and non-aqueous solvents on enzyme activity are well-documented. In aqueous solvents, which are more native to enzymatic processes, water acts as a nucleophile towards enzymes. For example, in serine peptidases, water resolves acyl-enzyme intermediates and reforms the catalytic triad (9). Non-aqueous solvents change the physical properties of the solution, such as dielectric charge, polarity, and hydrophobicity, which would, in turn, affect acyl-enzyme stability and the rate of enzymatic hydrolysis. The impacts of organic solvents on the catalytic activity of enzymes can be attributed to changes in reactive intermediates in the active site or alterations in the noncovalent interactions that define an enzyme's secondary and tertiary structure (10). Macromolecular stability, defined as little-to-no structural changes, can be studied using molecular dynamics—a biophysics tool used to link protein structure and its dynamics—which allows us to determine the stability of lipase in non-aqueous solvents (11, 12).

Over the past decades, numerous colorimetric methods have been used to probe enzymatic activity: peroxidase activity is measured by the reduction of diamines, protease activity is measured using nitroanilide substrates, and esterase activity is measured using nitrophenyl esters (13-15). 4-nitrophenyl acetate is a commonly used substrate to determine lipase activity (16). Enzymatic cleavage of the nitrophenyl ester bond by lipases releases nitrophenol, which can be tracked using UV-visible (UV-vis) spectroscopy, a technique measuring the amount of light that a substance absorbs at a specific wavelength, via absorbance at a wavelength of 413 nanometers (nm) (**Figure 1C**).

Here, we synthesized 4-nitrophenyl acetate from the acylation of 4-nitrophenol and utilize UV-vis spectroscopy to screen and quantify lipase activity in seven organic solvents using 4-nitrophenyl acetate as a colorimetric probe. We hypothesize that the greatest retention of enzymatic activity would be observed in solvent systems whose physical properties most closely mimic water and that higher concentrations of non-aqueous solvent will decrease enzymatic activity. The common organic solvents screened were chosen based on their miscibility with water: methanol, ethanol, isopropanol, acetonitrile, acetone, N,N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO). Lipase activity was monitored at 5%, 10%, 20%, and 40% concentrations of each organic solvent. While we determined that lipase activity decreased with increasing concentrations of organic solvent, as hypothesized, lipase maintained the highest rate of activity in 5% methanol, suggesting that out of all solvents tested, an aqueous methanol solvent system could be the most favorable in which to perform deacylation reactions involving lipase and organic compounds.

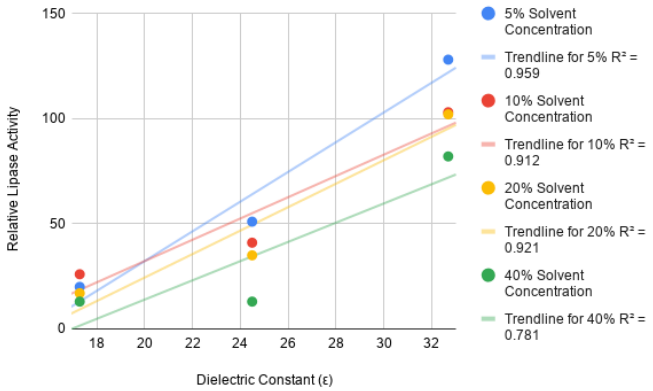

Figure 3. Alcohol polarity impact on lipase activity. Each line represents a different concentration of solvent (blue = 5%; red = 10%; yellow = 20%; green = 40%), and each alcohol is represented by its dielectric constant (ε 17.9 = isopropanol; ε 24.5 = ethanol; ε 32.7 = methanol).

RESULTS

UV-Vis data of several 4-nitrophenol solutions were used to define extinction coefficients, which allowed us to calculate the concentration of nitrophenol as a function of absorbance. In order to measure lipase activities, we measured the accumulation of 4-nitrophenol over time and compared results to those of aqueous lipase. Consistent with our hypothesis, relative lipase activity (compared to lipase activity in water) generally decreased in higher concentrations of organic solvent (**Figure 2**). This loss of enzymatic activity was most apparent in methanol, where an increase in methanol concentration from 5% to 40% caused a decrease of relative enzymatic activity by 46%.

Contrary to our initial expectations, lipase had higher relative levels of activity in lower concentrations of methanol than in water, with 128% lipase activity at 5% methanol, 103% lipase activity at 10% methanol, and 102% enzyme activity at 20% methanol. Lipase activity for all other organic solvents was lower than that of water at all concentrations. A solvent system of 40% acetone resulted in the largest decrease in lipase activity, with only 1% of lipase activity compared to lipase in water. Increasing concentrations of methanol also resulted in the largest decrease in relative lipase activity of 46% between 5% and 40% methanol. On the other hand, increasing concentrations of isopropanol resulted in the smallest decrease in relative lipase activity of 7% between 5% and 40% isopropanol.

We also observed that alcohol solvents (methanol, ethanol, and isopropanol) tend to follow a trend in which a decrease in polarity, or a decrease in the dielectric charge, corresponds to a decrease in lipase activity (**Figure 3**). Methanol, being the most polar of the solvents screened, displayed the highest relative lipase activity, followed by ethanol and isopropanol (least polar). Methanol is the most polar of the three and has properties most similar to water, such as similar dipole properties. Therefore, its dipole interactions with lipase most closely mimic those of water.

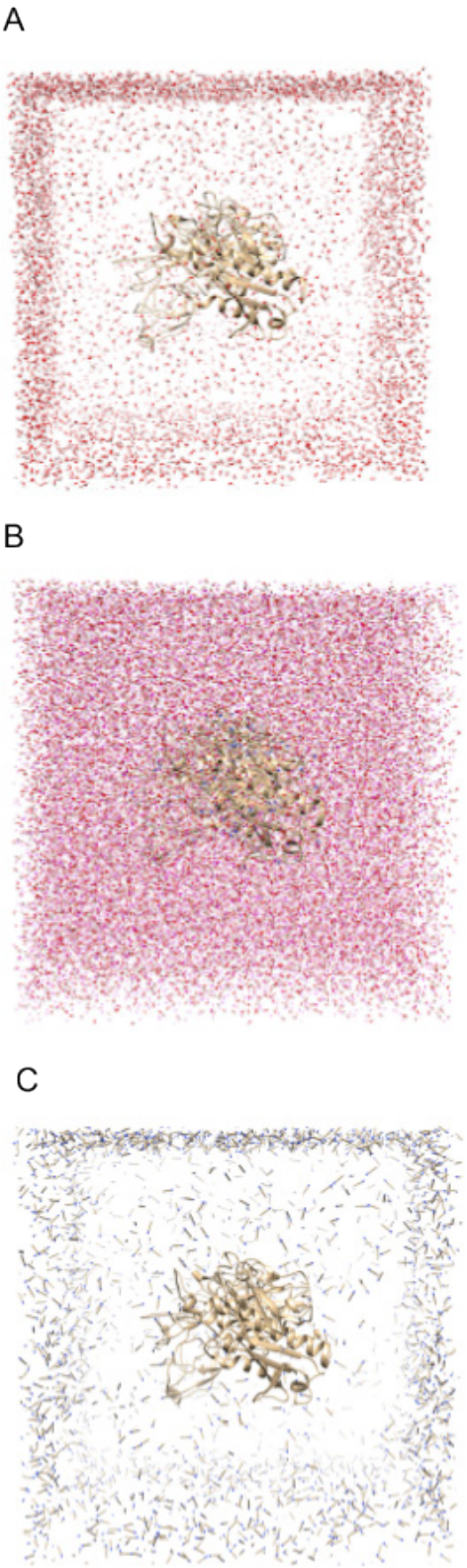

Figure 4. Structures of Lipase in Various Solvents. (**A**) Structure of lipase in water solvation shell. Red represents the solvation shell as water molecules. (**B**) Structure of lipase in methanol solvation shells. The red represen(ts the solvation shell as methanol molecules. (**C**) Structure of lipase in acetonitrile solvation shell. Red represents the solvation shell as acetonitrile molecules.

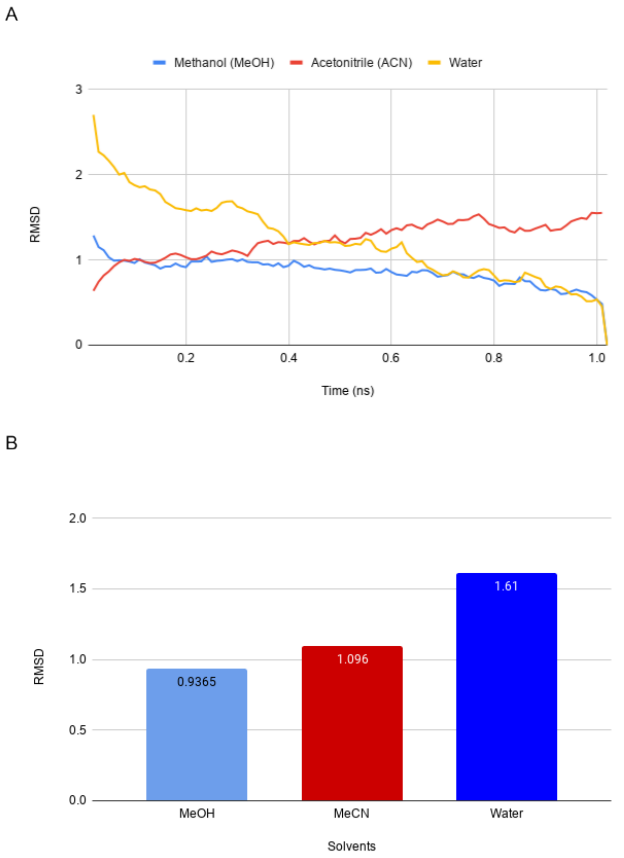

Figure 5. Root mean square deviation (RMSD) Values of Lipase in Various Solvents. RMSD is the deviation of atomic positions between the atoms of the lipase. (**A**) RMSD values of lipase graphed as a function of time in methanol, acetonitrile, and water solvent systems. (**B**) Average RMSD values of lipase in methanol, acetonitrile, and water. Average calculated as the average RMSD values overtime.

Other notable observations are the relatively unchanged rates of lipase activity despite increased concentrations of DMSO. There were also decreases in lipase activity after increases in the concentration of DMF and acetonitrile (**Figure 2**).

Molecular dynamics simulations were also performed using GROningen MAchine for Chemical Simulations (GROMACS) to probe predicted changes in lipase stability in water, methanol, and acetonitrile (**Figure 4**). We were unable to perform these calculations for every solvent because we could not obtain all solvent topology files. Root mean square deviation (RMSD) values for lipase were derived from GROMACS and graphed as a function of time (**Figure 5A**) as well as averages of the time dependent RMSD for each solvent (**Figure 5B**). Lower RMSDs indicate less deviation of atomic positions, between the atoms of the lipase, and analysis of this can suggest protein stability. Over time, the RMSD of lipase in acetonitrile increased, while the RMSD of water and methanol decreased. The average RMSD shows in which solvent lipase is predicted to be most stable versus unstable. Further examination showed lipase in methanol to have the lowest average RMSD of 0.9365 Å, while lipase in water has the highest average RMSD of 1.61 Å.

DISCUSSION

Here, we screened the kinetics of catalytic deacylation of 4-nitrophenyl acetate by a bovine pre-gastric lipase through UV-vis spectroscopy in different organic solvents in efforts to determine the extent of retention of lipase catalytic ability in non-aqueous solvent systems which helps with chemical synthesis under mild conditions. We observed a general decrease in lipase activity with increasing concentrations of non-aqueous solvent, which is consistent with our hypothesis that enzymatic activity would be best retained in solvent systems whose physical properties most closely mimic water and that higher concentrations of non-aqueous solvent will decrease enzymatic activity. However, in low concentrations of methanol, there was a significant increase in lipase activity relative to an aqueous system.

The relative lipase activity in acetone, acetonitrile, and DMF was lower as compared to lipase activity in water as evidenced by the slower increase in absorbance at 413 nm. Lower lipase activity in acetone, acetonitrile, and DMF can likely be attributed to solvent molecules disrupting crucial hydrogen bond interactions with lipase that may otherwise be present with an alcohol solvent. Similar to DMSO, these three solvents lack hydrogen-donor properties that both water and methanol possess, which likely affects the activity of lipase. For example, hydrogen bonds stabilize the tertiary structure of proteins; therefore, without hydrogen bonds, the tertiary structure of lipase may be affected, which would impact its activity. Decreased lipase activity in acetone may also be explained by the lower polarity of acetone compared to water or methanol. Since high catalytic activity in DMSO compared to DMF and acetonitrile cannot be rationalized on the basis of polarity, as DMSO is fairly more polar as compared to DMF and acetonitrile, it seems that loss of enzymatic activity in these solvents is most likely attributed to differences in the degree of disruption in the hydrogen bonding network that defines secondary and tertiary structure in the enzyme. Competition for amide hydrogen-bond networks have been reported with both DMSO and DMF (17).

Our MD simulations suggested that lipase destabilizes in water, as evidenced by lipase's high average RMSD in water. However, the lipase in water RMSD values start at higher values than the lipase in methanol RMSD values, yet they both ended in similar RMSD values. This suggests that the lipase stabilizes faster in methanol than it does in water. Lipase had the lowest average RMSD in methanol, which indicates that lipase experiences the least deviation in atomic positions in methanol compared to water and acetonitrile. Similarly, the time-dependent RMSD behavior of lipase in methanol was lower to that of lipase in water, suggesting that the conformational state and stability of the enzyme in methanol is higher than that of the enzyme in water. This is consistent with our spectroscopic data since lipase displayed higher enzymatic activity in low concentrations of methanol than in water. The RMSD of lipase in acetonitrile increased

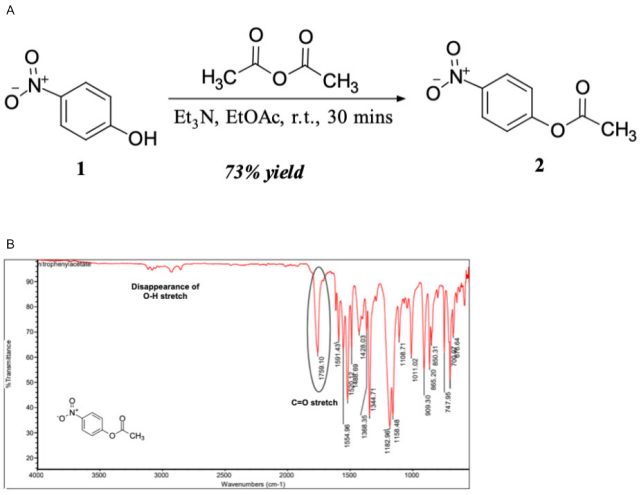

Figure 6. Reaction Schematic and Characterization of 4-nitrophenyl Acetate. (**A**) 4-nitrophenyl acetate [2] was synthesized in 73% yield via acetylation of 4-nitrophenol [1]. (**B**) FT-IR spectrum of 4-nitrophenyl acetate.

over time, which may be attributed to a net destabilization of key noncovalent interactions that define the secondary or tertiary structure of lipase. While further mechanistic studies would be needed to identify whether this translates into greater catalytic activity, these initial MD simulations seem to indicate that the three-dimensional stability of the lipase enzyme in methanol is more similar to its native aqueous environment, in comparison to a solvent such as acetonitrile, and this is consistent with the observation that lipase activity is highest in methanol compared to any other organic solvent.

It was observed that in 5% methanol, lipase activity was 28% higher compared to lipase activity in water. While low concentrations of methanol seem to accelerate the rate of enzymatic hydrolysis of the 4-nitrophenyl acetate ester bond, a trend of decreasing enzymatic activity with increasing concentrations of non-aqueous solvent was still observed. However, across all types and concentrations of organic solvent, enzymatic catalysis in methanol exhibited the greatest rate of substrate deacylation. Therefore, low concentration aqueous methanol solutions may be a desirable solvent system to use when conducting lipase-catalyzed deacylation reactions on organic compounds. Our results inform future efforts involving the optimization of water-methanol co-solvent systems in lipase deacylation reactions.

To further analyze enzymatic activity in non-aqueous organic solvents, future experimentation can be done with other esterases such as acetylcholinesterase, phosphatases, and endonucleases. In doing so, future research can be used to determine whether the trends observed in this study are unique to lipase or whether they represent a general trend for all esterases.

METHODS

Chemical Synthesis

4-nitrophenyl acetate was synthesized via acetylation of 4-nitrophenol. 4-nitrophenol (1.00g, 1 eq., 7.2 mmol) was added to a round-bottom flask equipped with a Teflon stir bar, along with acetic anhydride (11g, 15 eq., 107.8 mmol), triethylamine (0.73g, 1 eq., 7.2 mmol), in ethyl acetate. The progress of the reaction was monitored to completion via thin-layer chromatography. Unreacted acetic anhydride was quenched with methanol, and the crude material was concentrated in a rotary evaporator (Rotovap Buchi R200). The crude product was extracted 3 times in ethyl acetate over saturated sodium bicarbonate to remove excess acetic acid. The combined organic layers were dried over anhydrous magnesium sulfate, concentrated in vacuo and purified on silica gel flash chromatography with a gradient of 0-25% ethyl acetate in hexanes to yield off-white crystals of 4-nitrophenyl acetate with 73% yield (**Figure 6A**). 4-nitrophenyl acetate was characterized via 1H NMR (Nanalysis NMReady 60 MHz 1H NMR spectrometer in chloroform-D), Fourier-transform infrared (FT-IR) spectroscopy (Thermo Scientific iS5 Nicolet FT-IR spectrometer, iD5 ATR assembly) (**Figure 6B**), and UV-vis spectroscopy (BioRad SmartSpec 3000 UV-vis spectrophotometer, quartz cuvette). 4-nitrophenol was characterized via FT-IR and UV-vis spectroscopy. American Chemical Society (ACS) grade 4-nitrophenol was purchased from Reagent Inc. and used without further purification. ACS grade acetic anhydride was purchased from ChemSavers Inc. All solvents used were ACS grade.

4-nitrophenyl acetate: (^{1}H NMR, 60 MHz, CDCl3): δ 7.21-8.34 (dd, 4H, J = 8.3Hz), 2.35 (s, 3H); FT-IR (ATR, cm^{-1}): 2900-3100 (Ar-H), 1759.10 (C=O), 1591.43, 1554.96, 1520.12, 1368.35.
4-nitrophenol: FT-IR (ATR, cm-^{1}): 3317.81 (O-H), 2900-3100 (Ar-H), 1612.64, 1586.58, 1486.66, 1319.95.

Preparation of Enzyme Solutions

Enzyme buffers at pH 8 were prepared with 15 mM Tris base and 100 mM sodium chloride in deionized water. The Tris buffer was used because of its availability and ability to buffer the enzyme solution to pH 8, the optimal pH of lipase. Then, solvent solutions of concentrations 5%, 10%, 20%, and 40% were prepared for each solvent (DMSO, Ethanol, Methanol, Isopropanol, Acetone, DMF, and Acetonitrile) in a buffer. To compare the lipase activity of the lipase in solvents we also monitored the hydrolysis kinetics of the lipase in 100% water. Bovine pre-gastric lipase, obtained as a commercially-available powder from Standing Stone Farms, was then dissolved in each of the solutions to produce a (0.5 mg/mL) solution. To our knowledge, there are no significant differences between human and bovine lipases.

UV-Vis Spectrophotometry

A full UV-vis spectrum of 4-nitrophenol revealed two peaks, one around 413 nm and one at 310 nm, while a UV-Vis spectrum of 4-nitrophenyl acetate revealed a peak at around 300 nm. We chose to track 4-nitrophenol at 413

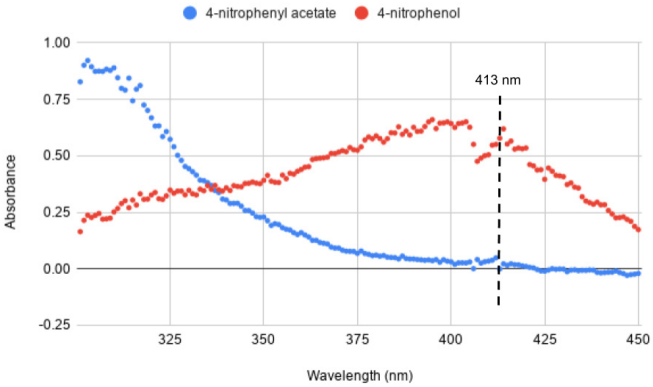

Figure 7. Final average spectrum of overlaid UV-vis spectra of 4-nitrophenol (blue) and 4-nitrophenyl acetate (red).

nm because 4-nitrophenyl acetate does not absorb at that wavelength, which allowed for much simpler calculations when determining the hydrolysis kinetics of lipase (Figure 7). The extinction coefficients of 4-nitrophenol at 413 nm were determined via Beer's Law for each solvent system sampled.

A 1 mM solution of 4-nitrophenyl acetate in 5% DMSO (for the dissolution of nitrophenyl acetate) was prepared. 1 mL of substrate solution and 1 mL of a buffered enzyme solution were mixed in a cuvette. To ensure that observed changes in absorbance during the experiments were a result of enzymatic hydrolysis, separate controls of substrate-only (no-enzyme) and enzyme-only solutions were prepared. The cuvettes were placed in a UV-vis spectrophotometer and tracked at 413 nm every 10 seconds for 3 minutes. All solutions were run immediately after preparation.

We analyzed UV-vis data to determine respective Beer's Law graphs of 4-nitrophenol at 1.00 nM, 10.0 nM, and 50.0 nM. Extinction coefficients were derived from Beer's Law graphs and used to calculate the concentration of nitrophenol as a function of absorbance at 413 nm. The rate of enzymatic hydrolysis is measured using nitrophenyl acetate in each solvent was obtained by determining the initial velocity of lipase, which was calculated using the slope of the line-of-best-fit of the concentration of 4-nitrophenol as a function of time (**Figure 7**).

Molecular Dynamics

To quantify the stability of the enzyme in a non-aqueous environment, molecular dynamics simulations were performed. Molecular dynamics simulations of lipase (PDB: 1N8S) were conducted using GROMACS (18, 19). Solvent models for methanol and acetonitrile were used from the GROMACS molecule topologies website, and the water model was used directly from the software (19). The GROMOS96 43a2 forcefield was used, and the lipase was placed in a cubic solvent box at a distance of 1.0 nm from the solvent molecules. Energy minimization was then run to minimize the structure and clear clashes between the solvent atoms and lipase. Equilibration was run in two parts to reach the thermodynamic requirements of the structure. The final step was run in 50000 steps using Berendsen pressure coupling.

Molecular dynamics simulations were performed on a Dell Poweredge 710 server with a 24 core Intel Xeon X5660 processor @ 2.80GHz and 32GB RAM. Results were analyzed using Visual Molecular Dynamics and MDWeb Server (20, 21).

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