Liquid-Liquid Phase Separation Regulates Enzymatic Activity

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ABSTRACT

Cellular crowding plays a key role in regulating the enzymatic reactivity, which is challenging to realize in the dilute phase. However, the molecular interpretation behind this enhanced reactivity is poorly understood. Herein, using horseradish peroxidase (HRP) and glucose oxidase (GOx) cascade pair, we demonstrate for the first time that macromolecular crowding induces liquid-liquid phase separation via liquid-like droplet formation and thereby increases the intrinsic catalytic efficiencies of HRP and GOx. Our findings reveal unprecedented enhancement (18–338-fold) in the catalytic efficiency within the droplet phase relative to the bulk aqueous phase. The present discovery highlights the active role of liquid-like membraneless condensates in regulating various complex metabolic reactions under crowded cellular environments.

1. INTRODUCTION

Cellular metabolic reactions are considerably more complex yet highly efficient than the in vitro reactions under dilute buffer conditions.[1] How the complex and heterogeneous cellular environment regulates various complex biochemical reactions with high specificity and selectivity is a fundamental question in cell physiology, which has not been understood yet. The cellular interior is highly crowded (~200–400 g/L) with various bio-macromolecules which occupy ~40% of its volume.[2] Earlier, it has been demonstrated that macromolecular crowding (MC) plays a key role in regulating various fundamental processes including protein folding,[3–5] aggregation,[6] diffusion,[7,8] thermal stability,[9] DNA replication,[10] and enzyme kinetics.[11–21] In particular, the effect of crowding on enzymatic activity is complex and difficult to predict. While some enzymes display enhanced activity[12,14,20] in the presence of crowders, the catalytic rates of others are either decreased[11,13,15,17] or remain unaltered.[22] Similarly, crowding may also modulate the Michaelis-Menten constant, K_m by either lowering[12,13,15] or increasing[8] its value compared to that in dilute buffer conditions. In contrast, few earlier studies reported unaltered values of K_m upon crowding.[11,22] The increase in K_m is accounted by considering diffusion resistance in a crowded environment, while the reduction is attributed to changes in activity coefficients. These contradictory trends of kinetic parameters indicate the complex role of MC on the enzymatic reactions.

In spite of these earlier efforts, the fundamental mechanism behind the activity enhancement upon crowding is still not clear. In general, the activity enhancement was accounted by considering stabilization of active conformation of enzymes via excluded volume effect (EVE) under a crowded milieu. However, apart from the classical EVE, crowding can also modulate the soft protein-protein (SPP) interactions via weak nonspecific chemical (NC) interactions.[23–28] While the EVE is mainly entropic in nature and often stabilizes the compact native protein structure, NC interactions are enthalpic in nature and can either stabilize or destabilize the native protein structure.

Another important aspect of MC is its ability to induce liquid-liquid phase separation (LLPS) of many disease-associated proteins containing intrinsically disordered regions (IDRs) with low complexity domains (LCDs) in their amino acid sequence via membraneless droplet formation.[29–33] Very recently, we along with Maji and coworkers independently discovered that even globular proteins lacking any IDRs could also undergo LLPS upon crowding via SPP interactions.[34,35] Although these recent findings delineate the role of SPP interactions on the physiological behavior of proteins, their impact on enzymatic activity is highly neglected in crowded environments. Inspired from our recent finding, we envisaged that soft intermolecular interactions between enzymes under a crowded milieu could potentially dominate over classical EVE and may provide an alternate metabolic pathway. Here we examined the catalytic activity of two well-known model enzymes, namely horseradish peroxidase (HRP) and glucose oxidase (GOx) in the absence and presence of inert synthetic crowder polyethylene glycol (PEG 8000) (Figure 1A), as their catalytic pathways were well explored in the literature.[36,37] More importantly, GOx/HRP cascade pair has found widespread applications in various fields. This pair catalyses the oxidation of glucose by GOx in the presence of oxygen (O_2) to produce hydrogen peroxide (H_2O_2), which is subsequently utilized by HRP to oxidize organic or inorganic substrates by releasing O_2 (Figure 1A). HRP belongs to the superfamily of heme-containing plant peroxidases with a molecular weight of 44 kDa and consists of a single polypeptide chain with 308 amino acid residues.[38] On the other hand, GOx is a homodimer and belongs to the oxidoreductase family with a molecular weight of 160 kDa and consists of 605 amino acid residues.[39]

2. RESULTS AND DISCUSSION

Initially, we tested the influence of 10% PEG 8000 on the individual kinetic parameters of HRP and GOx. The kinetics of both the enzymes was monitored using a UV-vis spectrophotometer (see the SI for details). The enzymatic rate of 25 pM HRP in the presence of 8.8 mM H_2O_2 in aqueous acetate buffer (pH 4) at 37 °C follows a typical Michaelis-Menten plot as a function of TMB concentrations (Figure

1B and S1). Similar plots are also observed with other substrates, namely ABTS and OPD (Figures S2 and S3). The estimated Michaelis-Menten constants (K_m) and turnover numbers (k_{cat}) are tabulated in Table 1.

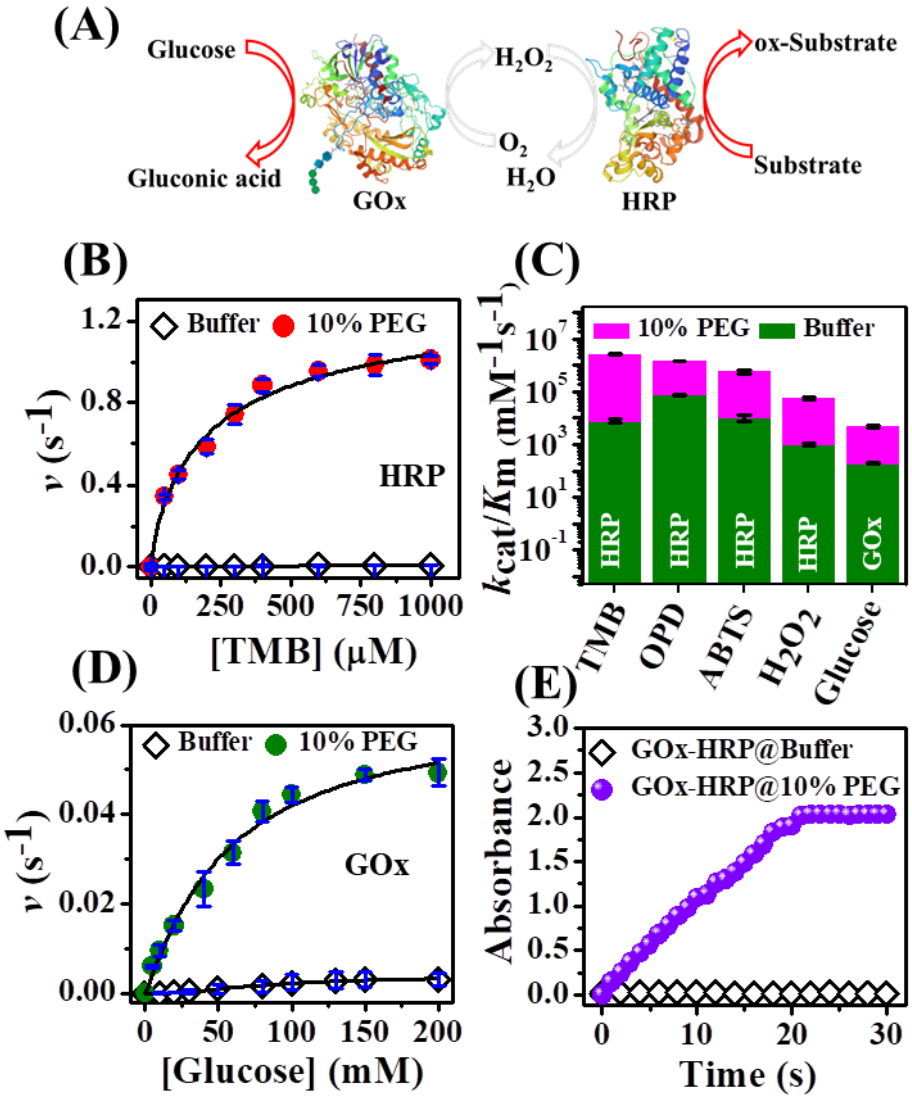


Figure 1. (A) Crystal structure of GOx (PDB entry 1CF3) and HRP (PDB entry 1H58) along with their metabolic reactions. Effect of PEG on (B) Michaelis-Menten plot of HRP, (C) k_{cat}/K_m of HRP and GOx for different substrates, (D) Michaelis-Menten plot of GOx, and (E) Absorbance of ox-TMB at 650 nm as a function of time for GOx/HRP cascade reaction.

The K_m and k_{cat} values for H_2O_2 were estimated by keeping the concentration of TMB fixed at 1 mM (Figure S4 and Table 1). These parameters are consistent with prior reports.[40] In contrast, the cata-

lytic rates increase dramatically as a function of substrate concentrations in the presence of PEG (Figures 1B, S5, and S6). The estimated kinetic parameters in the presence of PEG reveal almost unchanged values of K_m relative to those estimated in aqueous buffer (Table 1).

Table 1. Estimated Kinetic Parameters

Enzyme	Substrate	Buffer		10% PEG	
		K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
HRP	TMB	0.5	4.0×10^3	0.3	5.4×10^5
	ABTS	0.4	4.0×10^3	0.2	1.2×10^5
	OPD	0.3	2.3×10^4	0.2	2.3×10^5
	H_2O_2	3.4	3.5×10^3	3.7	2.1×10^5
GOx	Glucose	81.4	1.5×10^4	57.0	2.6×10^5

Remarkably, the k_{cat} for TMB increases by 135-fold, whereas it increases by 30-, 10-, and 60-fold for ABTS, OPD, and H_2O_2, respectively. Notably, the catalytic efficiency (k_{cat}/K_m) in the presence of PEG increases by 338-, 60-, 18-, and 57-fold for TMB, ABTS, OPD, and H_2O_2, respectively (Figure 1C). These findings suggest that crowding has tremendous influence on the enzymatic activity of HRP. A control experiment with 100-fold molar excess of HRP in buffer reveals that the observed enhancement is not due to a simple mass action mechanism (Figure S7). On the other hand, the estimated K_m of GOx decreases from 81.4 mM in PBS (pH 7.4) to 57.0 mM in the presence of PEG,[41] whereas the k_{cat} increases by 17.3-fold from 1.5×10^4 to 2.6×10^5 s^{-1} (Figure 1D, Table 1). The decrease in K_m upon crowding indicates higher affinity of GOx towards glucose. Next, we examined the influence of crowding on the efficiency of the GOx/HRP cascade reaction (see the SI for details). In buffer, the apparent rate constant (k_{app}) of the cascade reaction is estimated to be 3.1×10^{-3} s^{-1}, which increases to 1.1×10^{-1}

s^{-1} in the presence of 10% PEG (Figures 1E and S8). This 37-fold enhancement in the k_{app} value of the GOx/HRP cascade reaction is unprecedented in the literature and indicates the active role of crowding on the cascade activity.

To know the conformational perturbation, we performed circular dichroism (CD) measurements. The far-UV CD spectrum of HRP at pH 4.0 displays two minima at 208 and 222 nm, suggesting an α-helical rich secondary structure (Figure 2A). However, a distinct red-shift of 4 nm with an increase in ellipticity at 222 nm has been observed in the presence of PEG. Similarly, the CD spectrum at the Soret region and UV-vis spectrum of HRP in the presence of PEG display a prominent red shift of 3 and 15 nm, respectively compared to that in buffer (Figure 2B,C). These spectral change of HRP upon crowding indicates more compact secondary structure and higher integrity of the heme pocket.[42] In contrast, the ellipticity at 222 nm decreases slightly upon crowding (Figure 2D), suggesting a subtle conformational change of GOx. Taken together, our findings clearly authenticate crowding-induced conformational alteration of both HRP and GOx. Next, we seek to address how these conformational changes of HRP and GOx under crowding affect the SPP interactions in solution by using confocal laser scanning microscopy (CLSM) and fieldemission scanning electron microscopy (FESEM). CLSM measurements were performed using 0.5 μM fluorescently labeled proteins (see SI for more details). Surprisingly, both the enzymes in the presence of 10% PEG at 37 °C display uniform spherical morphologies as revealed from confocal differential interference contrast (DIC) images (Figure 3A). These spherical structures exhibit liquid-like droplet characteristics such as fusion, dripping, and surface wetting (Figures 3A and S9), similar to those observed earlier for other proteins.[29–33] Notably, no droplets have been observed in the absence of PEG, indicating the active role of crowding behind this phenomenon (Figure S10).

CLSM images with RBITC-labeled HRP and FITC-labeled GOx confirm the presence of enzymes inside these droplets even at a working concentration of 25 pM (Figures 3B and S11). It is note-

worthy to mention that fluorescent labeling does not perturb the LLSP of the enzymes as revealed from our control experiments (Figure S12). Notably, droplet formation has also been observed in the presence of 12.5% Ficoll 400 and 10% Dextran 70, suggesting a general crowding effect (Figure 3B).

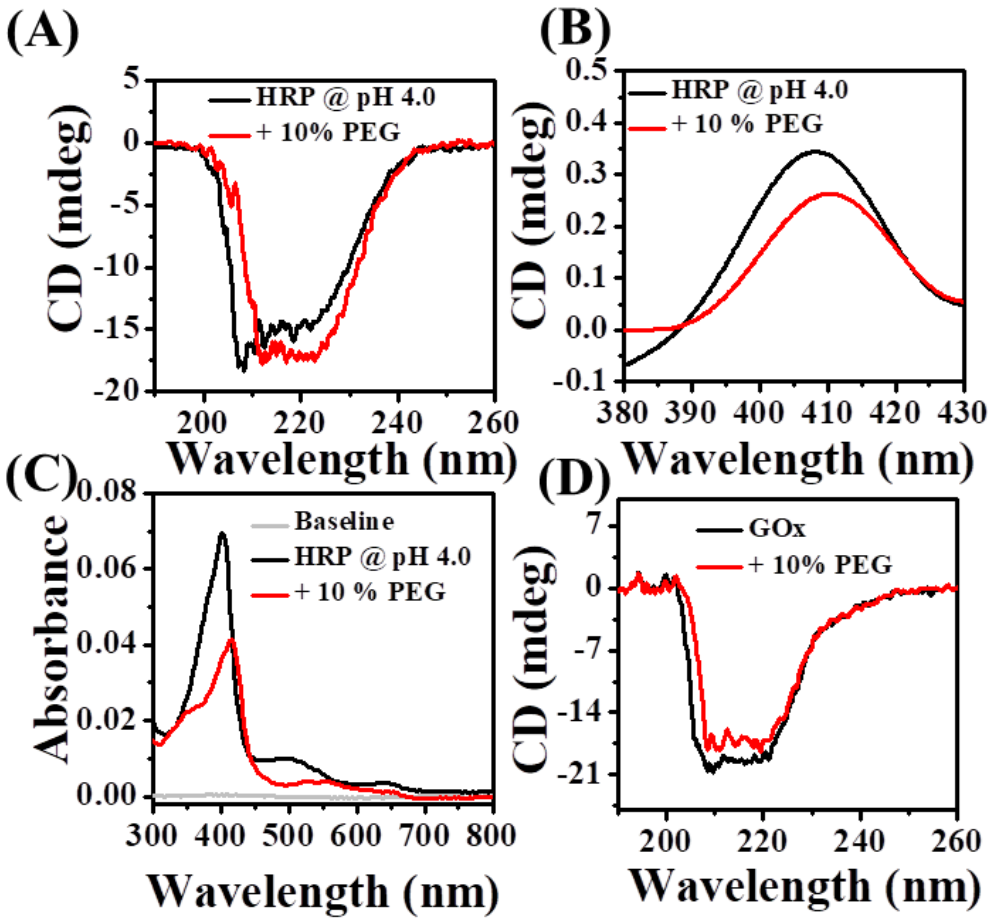


Figure 2. (A) Changes in the far-UV and (B) Soret region CD spectra of 0.5 μM HRP at pH 4 in the presence of PEG. (C) UV-vis absorption spectra of 0.5 μM HRP at pH 4 in the absence and presence of PEG. (D) Changes in the far-UV CD spectra of 0.5 μM GOx at pH 7.4 in the presence of PEG. All the measurements were performed at 37 °C.

Similar droplets have also been observed in FESEM measurements (Figure S13). These droplets are colloidally stable over a period of 15 days without any structural distortion (Figure S14). Interestingly, sequence prediction algorithms[43,44] reveal three LCDs (residues 4–17, 47–61, and 363–371) with six short

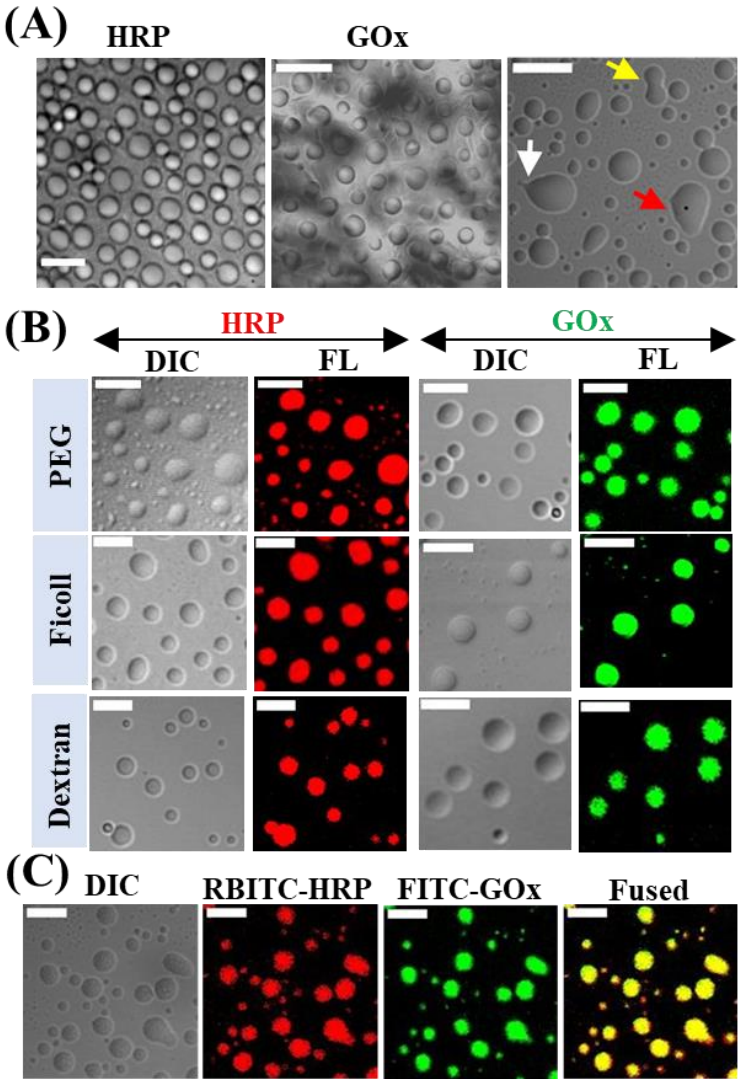


Figure 3. (A) DIC images of HRP and GOx droplets along with the DIC image of HRP showing coalescence (yellow arrow), dripping (white arrow), and surface wetting (red arrow) phenomena. (B) Confocal images of RBITC-labeled HRP and FITC-labeled GOx droplets formed in the presence of PEG, Ficoll, and Dextran. (C) Spontaneous coalescence between RBITC-labeled HRP droplets and FITC-labeled GOx droplets. The scale bars correspond to 5 μm.

disorder regions (residues 127–130, 192–204, 222–226, 237–243, 287–291, and 359–360) for GOx, whereas neither LCDs nor any disorder regions are predicted for HRP (Figure S15), suggesting that the presence of LCDs and/or IDRs is not an essential prerequisite for LLPS. Considering the isoelectric

point (pI) of HRP (8.8) and GOx (4.2), it is expected that the droplet formed by HRP at pH 4 will have a net positive charge, whereas droplet formed by GOx at pH 7 will bears a net negative charge.[45] To our surprise, when red-emissive RBITC-labeled HRP and green-emissive FITC-labeled GOx were mixed with 10% PEG, we observed uniformly distributed yellow emissive droplets within 15 min of their mixing, suggesting spontaneous coalescence between oppositely charged droplets of HRP and GOx (Figure 3C). This observation strongly suggests coalescence driven highly regulated cascade reaction via efficient substrate channeling within the fused droplets.

Droplet formation via LLPS of biomolecules is mainly driven by various weak multivalent intermolecular interactions including electrostatic and hydrophobic interactions.[29–33] To decipher the role of these interactions, we studied the droplet formation pathways as a function of pH, temperature, and ionic strength under CLSM. While HRP exhibits droplet formation in the pH range of 4–9, it is inhibited at pH 10 (Figures 4A and S16). Similarly, the droplets of GOx are stable in the pH range of 7.4–9; however, inhibited at pH values of 4 and 10 (Figures 4A and S17). These findings indicate the active role of charged residues of both the enzymes behind the droplet formation mechanism by considering their respective pI values. Moreover, HRP and GOx droplets are stable in the temperature range of 4–80 °C; however, inhibited completely at 90 °C (Figures 4B and S18), suggesting an upper critical solution temperature (UCST) profile.[34] The observed UCST profiles indicate a dominant role of enthalpically driven intermolecular interactions over the entropy of mixing as described previously by the Flory–Huggins theory.[46,47]

Next, we tested the feasibility of LLPS in the presence of neutral salt, sodium chloride (NaCl), chaotropic salt, sodium thiocyanate (NaSCN), and an aliphatic alcohol, 1,6-hexanediol. Droplet formation of both the enzymes in the presence of PEG is completely inhibited in the presence of 3 M NaCl (Figures 4C and S19), indicating the key role of strong electrostatic intermolecular interactions behind the LLPS of both the enzymes.[30,31,34] In contrast, droplets remain intact even at high concentrations of

1,6-hexanediol (6%) and NaSCN (1 M) (Figures 4D,E, and S20), suggesting an insignificant role of hydrophobic interactions.[30,34] Taken together, our findings illustrate that droplet formation is primarily driven by multivalent electrostatic intermolecular interactions between short patches of oppositely charged polypeptide chains.

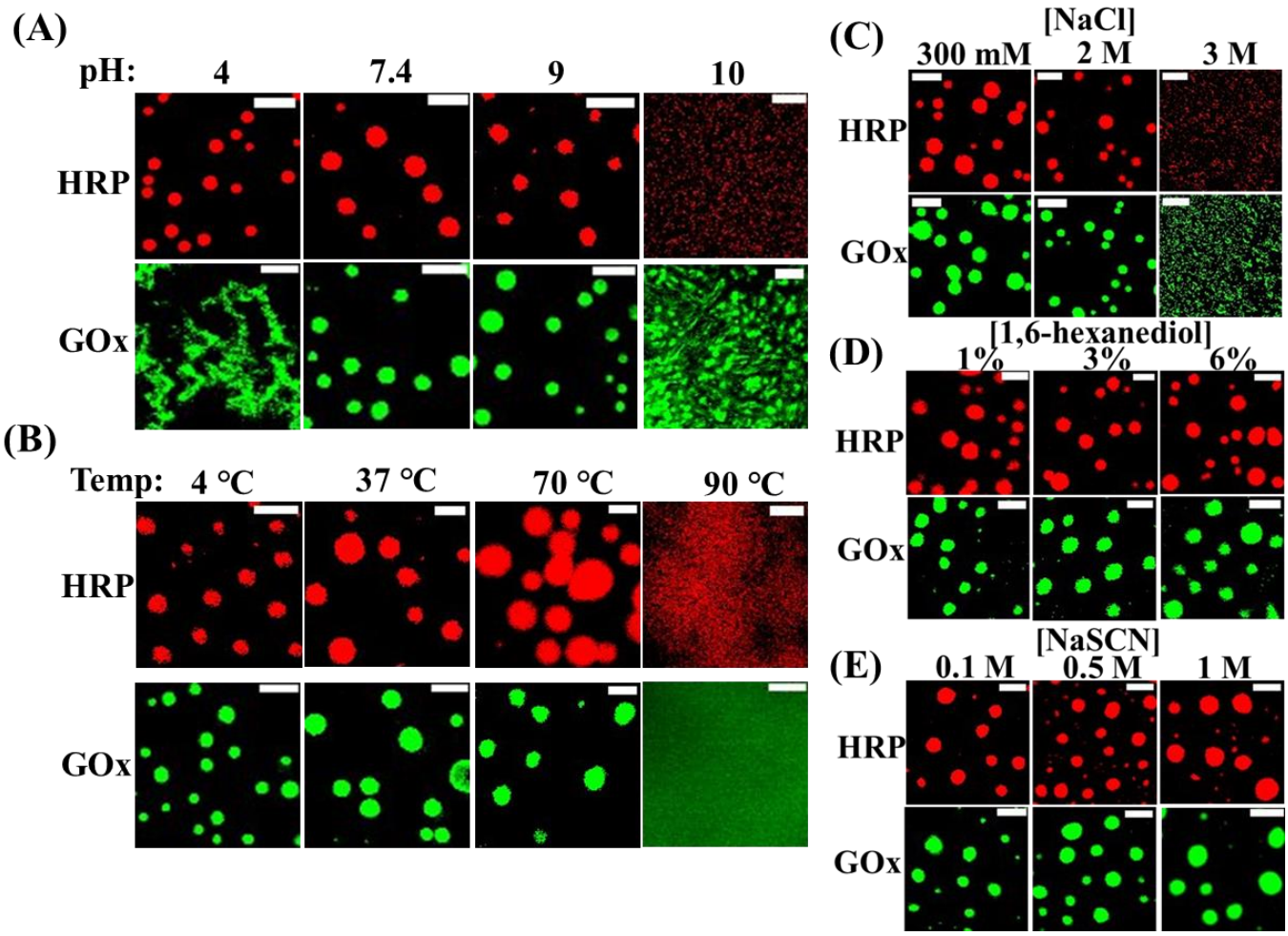
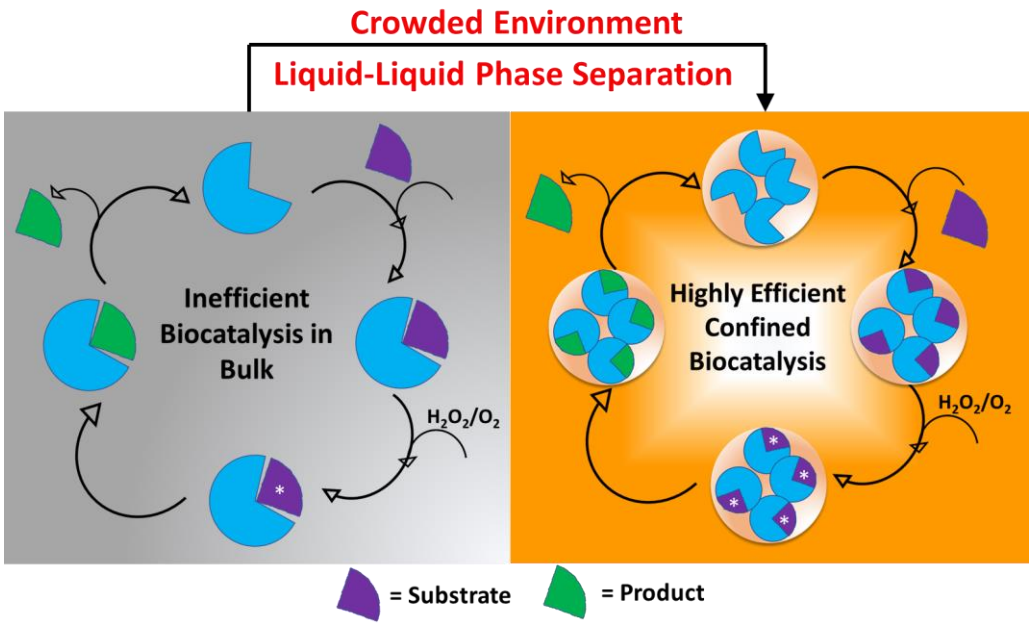


Figure 4. Confocal images showing the droplet stability as a function of (A) pH, (B) Temperature, (C) NaCl concentrations, (D) 1,6-hexanediol concentrations, and (E) NaSCN concentrations. The scale bars correspond to 5 µm.

On the basis of these findings, we present a mechanistic model where LLPS of enzymes via droplet formation under a crowded milieu amplifies the catalytic efficiencies of metabolic reactions

which are otherwise inefficient in an aqueous medium (Scheme 1). Further studies are currently underway in our group to explore this phenomenon for other enzymes in a crowded milieu.

Scheme 1. Regulation of Enzymatic Activity via LLPS



3. SUMMARY

In summary, we have discovered a unique and unprecedented phenomenon behind the crowding-induced enhancement of enzymatic activity via liquid-like droplet formation under physiological conditions. Our mechanistic model represents a first proof of concept example of enhanced enzymatic activity inside the phase separated liquid-like droplets under crowded milieu. Kinetic analyses revealed an unprecedented enhancement of catalytic efficiency (18–338-fold) inside the phase separated droplets depending on the nature of substrates. Moreover, mechanistic investigations revealed that LLPS of both the enzymes is primarily driven by multivalent electrostatic interactions. We believe that the present study will provide a new dimension to understand various metabolic reactions under crowded milieu.

ASSOCIATED CONTENT

Supplementary Information

Experimental details and Figures S1–S20

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Minton, A. P. How Can Biochemical Reactions Within Cells Differ from Those in Test Tubes? *J. Cell Sci.* **2006**, *119*, 2863–2869.

(2) Zhou, H.-X.; Rivas, G.; Minton, A. P. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annu. Rev. Biophys.* **2008**, *37*, 375–397.

(3) Li, J.; Zhang, S.; Wang, C.-c.; Effacts of Macromolecular Crowding on the Refolding of Glucose-6-phosphate Dehydrogenase and Protein Disulfide Isomerase. *J. Biol. Chem.* **2001**, *276*, 34396–34401.

(4) Ådén, J.; Wittung-Stafshede, P. Folding of an Unfolded Protein by Macromolecular Crowding in Vitro. *Biochemistry* **2014**, *53*, 2271–2277.

(5) Gomez, D.; Huber, K.; Klumpp, S. On Protein Folding in Crowded Conditions. *J. Phys. Chem. Lett.* **2019**, *10*, 7650–7656.

(6) White, D. A.; Buell, A. K.; Knowles, T. P. J.; Welland, M. E.; Dobson, C. M. Protein Aggregation in Crowded Environments. *J. Am. Chem. Soc.* **2010**, *132*, 5170–5175.

(7) Wenner, J. R.; Bloomfield, V. A. Crowding Effects on EcoRV Kinetics and Binding. *Biophys. J.* **1999**, *77*, 3234–3241.

(8) Pastor, I.; Vilaseca, E.; Madurga, S.; Garces, J. L.; Cascante, M.; Mas, F. Diffusion of Alpha-chymotrypsin in Solution-Crowded Media. A Fluorescence Recovery after Photobleaching Study. *J. Phys. Chem. B* **2010**, *114*, 4028–4034.

(9) Stagg, L.; Zhang, S.-Q.; Cheung, M. S.; Wittung-Stafshede, P, Molecular Crowding Enhances Native Structure and Stability of *α/β* Protein Flavodoxin. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 18976–18981.

(10) Akabayov, B.; Akabayov, S. R.; Lee, S.-J.; Wagner, G.; Richardson, C. C. Impact of Macromo lecular Crowding on DNA Replication. *Nat. Commun.* **2013**, *4*, 1615.

(11) Asaad, N.; Engberts, J. B. F. N. Cytosol-Mimetic Chemistry: Kinetics of the Trypsin-Catalyzed Hydrolysis of p-Nitrophenyl Acetate upon Addition of Polyethylene Glycol and N-tert-Butyl Acetoacetamide. *J. Am. Chem. Soc.* **2003**, *125*, 6874–6875.

(12) Jiang, M.; Guo, Z. Effects of Macromolecular Crowding on the Intrinsic Catalytic Efficiency and Structure of Enterobactin-Specific Isochorismate Synthase. *J. Am. Chem. Soc.* **2007**, *129*, 730–731.

(13) Poggi, C. G.; Slade, K. M. Macromolecular Crowding and the Steady-State Kinetics of Malate

Dehydrogenase. *Biochemistry* **2015**, *54*, 260–267.

(14) Norris, M. G. S.; Malys, N. What is the True Enzyme Kinetics in the Biological System? An In vestigation of Macromolecular Crowding Effect upon Enzyme Kinetics of Glucose-6-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **2011**, *405*, 388–392.

(15) Schneider, S. H.; Lockwood, S. P.; Hargreaves, D. I.; Slade, D. J.; LoConte, M. A.; Logan, B. E.; McLaughlin, E. E.; Conroy, M. J.; Slade, K. M. Slowed Diffusion and Excluded Volume Both Contribute to the Effects of Macromolecular Crowding on Alcohol Dehydrogenase Steady-State Kinetics. *Biochemistry* **2015**, *54*, 5898–5906.

(16) Wilcox, A. E.; LoConte, A. A.; Slade, K. M. Effects of Macromolecular Crowding on Alcohol Dehydrogenase Activity Are Substrate-Dependent. *Biochemistry* **2016**, *55*, 3550–3558.

(17) Wilcox, X. E.; Ariola, A.; Jackson, J. R.; Slade, K. M. Overlap Concentration and the Effect of Macromolecular Crowding on Citrate Synthase Activity. *Biochemistry* **2020**, *59*, 1737–1746.

(18) Balcells, C.; Pastor, I.; Vilaseca, E.; Madurga, S.; Cascante, M.; Mas, F. Macromolecular Crowd ing Effect upon in Vitro Enzyme Kinetics: Mixed Activation-Diffusion Control of the Oxidation of NADH by Pyruvate Catalyzed by Lactate Dehydrogenase. *J. Phys. Chem. B.* **2014**, *118*, 4062–4068.

(19) Nakano, S.; Karimata, H. T.; Kitagawa, Y.; Sugimoto, N. Facilitation of RNA Enzyme Activity in the Molecular Crowding Media of Cosolutes. *J. Am. Chem. Soc.* **2009**, *131*, 16881–16888.

(20) Totani, K.; Ihara, Y.; Matsuo, I.; Ito, Y. Effects of Macromolecular Crowding on Glycoprotein Processing Enzymes. *J. Am. Chem. Soc.* **2008**, *130*, 2101–2107.

(21) Aumiller, W. M.; Jr.; Davis, B. W.; Hatzakis, E.; Keating, C. D. Interactions of Macromolecular Crowding Agents and Cosolutes with Small-Molecule Substrates: Effect on Horseradish Peroxid ase Activity with Two Different Substrates. *J. Phys. Chem. B* **2014**, *118*, 10624–10632.

(22) Vopel, T.; Makhatadze, G. I. Enzyme Activity in the Crowded Milieu. *PLoS One* **2012**, *7*, e39418.

(23) Benton, L. A.; Smith, A. E.; Young, G. B.; Pielak, G. J. Unexpected Effects of Macromolecular

Crowding o Protein Stability. *Biochemistry* **2012**, *51*, 9773–9775.

(24) Sarkar, M.; Lu, J.; Pielak, G. J. Protein Crowder Charge and Protein Stability. *Biochemistry* **2014**, *53*, 1601–1606.

(25) Guseman, A. J.; Pielak, G. J. Cosolute and Crowding Effects on a Side-By-Side Protein Dimer. *Biochemistry* **2017**, *56*, 971–976.

(26) Kim, Y. C.; Mittal, J. Crowding Induced Entropy-Enthalpy Compensation in Protein Association Equilibria. *Phys. Rev. Lett.* **2013**, *110*, 208102.

(27) Harada, R.; Tochio, N.; Kigawa, T.; Sugita, Y.; Feig, M. Reduced Native State Stability in Crowded Cellular Environment Due to Protein-Protein Interactions. *J. Am. Chem. Soc.* **2013**, *135*, 3696–3701.

(28) Johansson, H.; Jensen, M. R.; Gesmar, H.; Meier, S.; Vinther, J. M.; Keeler, C.; Hodsdon, M. E.; Led, J. J. Specific and Nonspecific Interactions in Ultraweak Protein-Protein Associations Re vealed by Solvent Paramagnetic Relaxation Enhancements. *J. Am. Chem. Soc.* **2014**, *136*, 10277– 10286.

(29) Molliex, A.; Temirov, J.; Lee, J.; Coughlin, M.; Kanagaraj, A. P.; Kim, H. J.; Mittag, T.; Taylor, J. P. Phase Separation by Low Complexity Domains Promotes Stress Granule Assembly and Drives Pathological Fibrillization. *Cell* **2015**, *163*, 123–133.

(30) Wegmann, S.; Eftekharzadeh, B.; Tepper, K.; Zoltowska, K. M.; Bennett, R. E.; Dujardin, S.; Laskowski, P. R.; Mackenzie, D.; Kamath, T.; Commins, C.; Vanderburg, C.; Roe, A. D.; Fan, Z.; Molliex, A. M.; Hernandez-Vega, A.; Muller, D.; Hyman, A. A.; Mandelkow, E.; Taylor, J. P.; Hyman, B. T. Tau Protein Liquid-Liquid Phase Separation Can Initiate Tau Aggregation. *EMBO J.* **2018**, *37*, e98049.

(31) Ambadipudi, S.; Biernat, J.; Riedel, D.; Mandelkow, E.; Zweckstetter, M. Liquid-Liquid Phase Separation of the Microtubule-Binding Repeats of the Alzheimer-Related Protein Tau. *Nat. Comm un.* **2017**, *8*, 275.

(32) Protter, D. S. W.; Rao, B. S.; Treeck, B. V.; Lin, Y.; Mizoue, L.; Rosen, M. K.; Parker, R. Intrinsic

ally Disordered Regions Can Contribute Promiscuous Interactions to RNP Granule Assembly. *Cell Rep.* **2018**, *22*, 1401–1412.

(33) Ray, S.; Singh, N.; Kumar, R.; Patel, K.; Pandey, S.; Datta, D.; Mahato, J.; Panigrahi, R.; Nav alkar, A.; Mehra, S.; Gadhe, L.; Chatterjee, D.; Sawner, A. S.; Maiti, S.; Bhatia, S.; Gerez, J. A.; Chowdhury, A.; Kumar, A.; Padinhateeri, R.; Riek, R.; Krishnamoorthy, G.; Maji, S. K. α-Synuclein Aggregation Nucleates through Liquid-Liquid Phase Separation. *Nat. Chem.* **2020**, *12*, 705–716.

(34) Patel, C. K.; Singh, S.; Saini, B.; Mukherjee, T. K. Macromolecular Crowding-Induced Unusual Liquid-Liquid Phase Separation of Human Serum Albumin via Soft Protein-Protein Interactions. *J. Phys. Chem. Lett.* **2022**, *13*, 3636–3644.

(35) Poudyal, M.; Patel, K.; Sawner, A. S.; Gadhe, L.; Kadu, P.; Datta, D.; Mukherjee, S.; Ray, S.; Na valkar, A.; Maiti, S.; Chatterjee, D.; Bera, R.; Gahlot, N.; Padinhateeri, R. Maji, S. K. Liquid Con densate is a Common State of Proteins and Polypeptides at the Regime of High Intermolecular In teractions. *bioRxiv* **2021**, DOI: 10.1101/2021.12.31.474648.

(36) Zhang, Y.; Tsitkov, S.; Hess, H. Proximity Does Not Contribute to Activity Enhancement in the Glucose Oxidase-Horseradish Peroxidase Cascade. *Nat. Commun.* **2016**, *7*, 13982.

(37) Wang, C.; Yue, L.; Willner, I. Controlling Biocatalytic Cascades with Enzyme-DNA Dynamic Networks. *Nat. Catal.* **2020**, *3*, 941–950.

(38) Mogharrab, N.; Ghourchian, H.; Amininasab, M. Structural Stabilization and Functional Im provement of Horseradish Peroxidase upon Modification of Accessible Lysines: Experiments and Simulation. *Biophys. J.* **2007**, *92*, 1192–1203.

(39) Marín- Navarro, J.; Roupain, N.; Talens-Perales, D.; Polaina, J. Identification and Structural Analysis of Amino Acid Substitutions that Increase the Stability and Activity of Aspergillus niger Glucose Oxidase. *PLoS One* **2015**, *10*, e0144289.

(40) Gao, L.; Zhuang, J.; Nie, L.; Zhang, J.; Zhang, Y.; Gu, N.; Wang, T.; Feng, J.; Yang, D.; Perrett, S.; Yan, X. Intrinsic Peroxidase-Like Activity of Ferromagnetic Nanoparticles. *Nat. Nanotechnol.*

2007, *2*, 577–583.

(41) Holland, J. T.; Lau, C.; Brozik, S.; Atanassov, P.; Banta, S. Engineering of Glucose Oxidase for Direct Electron Transfer via Site-Specific Gold Nanoparticle Conjugation. *J. Am. Chem. Soc.* **2011**, *133*, 19262–19265.

(42) Haddad, R. E.; Gazeau, S.; Pécaut, J.; Marchon, J.-C.; Medforth, C. J.; Shelnutt, J. A. Origin of the Red Shifts in the Optical Absorption Bands of Nonplanar Tetraalkylporphyrins. *J. Am. Chem. Soc.* **2003**, *125*, 1253–1268.

(43) Schultz, J.; Milpetz, F.; Bork, P.; Ponting, C. P. SMART, A Simple Molecular Architecture Re search Tool: Identification of Signaling Domains. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 5857–5864.

(44) Erdős, G.; Dosztányi, Z. Analyzing Protein Disorder with IUPred2A. *Curr. Protoc. Bioinform.* **2020**, *70*, e99.

(45) Zhao, Z.; Fu, J.; Dhakal, S.; Johnson-Buck, A.; Liu, M.; Zhang, T.; Woodbury, N. W.; Liu, Y.; Walter, N. G.; Yan, H. Nonocaged Enzymes with Enhanced Catalytic Activity and Increases Sta bility Against Protease Digestion. *Nat. Commun.* **2016**, *7*, 10619.

(46) Flory, P. J. Thermodynamics of High Polymer Solutions. *J. Chem. Phys.* **1942**, *10*, 51–61.

(47) Huggins, M. L. Some Properties of Solutions of Long-Chain Compounds. *J. Phys. Chem.* **1942**, *46*, 151–158.