Unusual Liquid-liquid Phase Separation of Lysozyme Aqueous Solutions at Physiological pH and Salt Concentration

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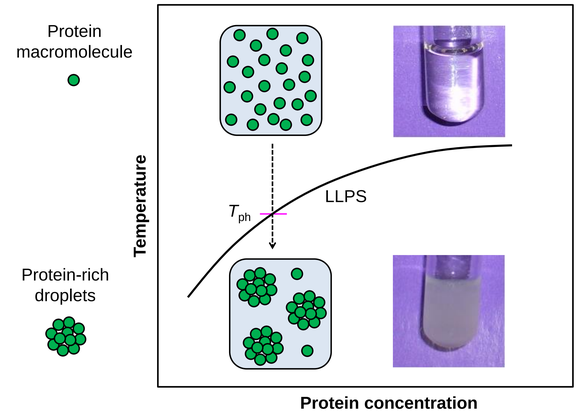
Introduction

Liquid-liquid phase separation (LLPS) of protein aqueous solutions is the *reversible condensation* of protein macromolecules into protein-rich micro droplets, driven by the net attractive interactions between protein molecules (Wang & Annunziata, *Langmuir*, **2008**, *24*, 2799-2807). LLPS occurs below a well-defined phase-transition temperature (T_{ph}).

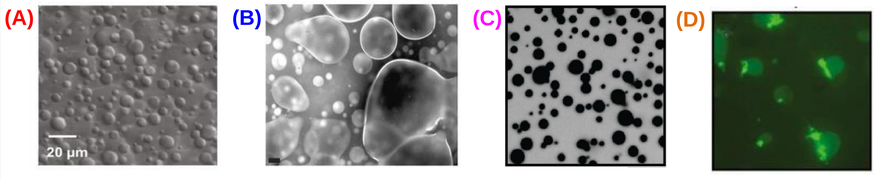


Above T_{ph}, a transparent protein solution is observed.

Below T_{ph}, protein-rich liquid droplets are formed and the mixture becomes turrbid.

Schematic temperature-concentration phase diagram of a protein aqueous system. Solid curve is the LLPS phase boundary.

Examples of recent LLPS studies relevant to Biological Systems

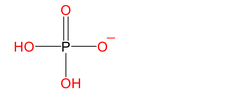


(A) RNA-binding protein hnRNPA1 (systemic amyloidosis diseases; *Science*, **2017**, *357*, eaaf4382). **(B)** HP1α protein (gene silencing; *Nature*, **2017**, *547*, 236-240). **(C)** Neurotransmitter protein Synapsin 1, (lipid vesicle trapping; *Science*, **2018**, *361*, 604-607). **(D)** Protein Tau (tauopathies; *The EMBO Journal*, **2018**, *37*, e98049).

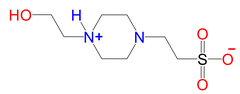
The effect of small molecules (osmolytes, ligands, buffer components) on the LLPS mechanism is not well understood. Since aqueous solutions of the protein lysozyme undergo LLPS at physiological composition (pH 7.4 and ionic strength ≈ 0.2) and T_{ph} ≈ 0 ºC , we investigated the effect of buffer type on the LLPS of this protein system. Here we show that replacing Tris or Phosphate buffers with another well-known buffer, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) significantly alters the LLPS mechanism. In contrast to the Tris and Phosphate buffer cases, the micro droplets produced in HEPES buffer at low temperature remain surprisingly stable upon heating even at ≈ 30 – 40 ºC.

Results and Discussion

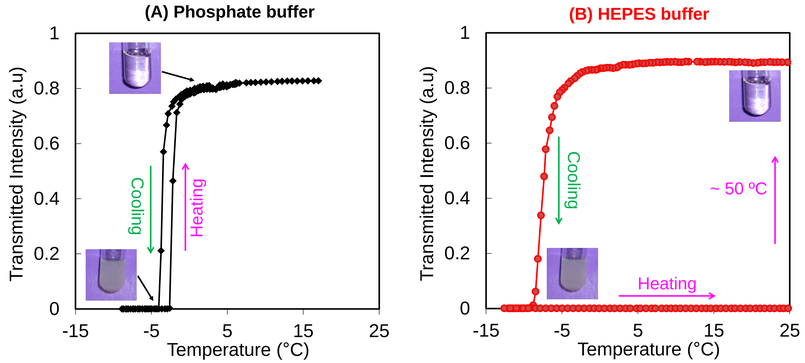
1. Phase Separation of Lysozyme in HEPES Buffer



Phosphate buffer p*K*a = 7.2



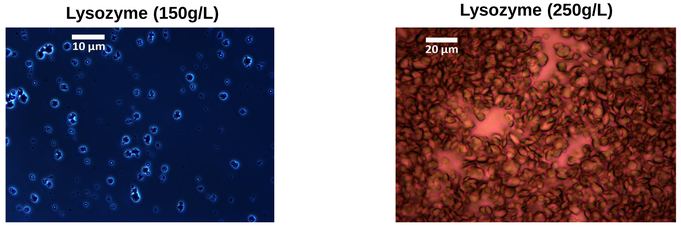
HEPES buffer p*K*a = 7.5



Representative transmitted intensity profiles for lysozyme solutions at pH 7.4. The buffer concentration is 0.1M and ionic strength is adjusted using NaCl to 0.2 M.

(A) Phosphate case: Sample opacification occurred at T_{ph} **-5 ºC** upon cooling and sample clarification occurred at **-4 ºC** upon heating.

(B) HEPES case: Sample opacification occurred at T_{ph} **-9 ºC** upon cooling while sample clarification was observed at **~50 ºC** upon heating.

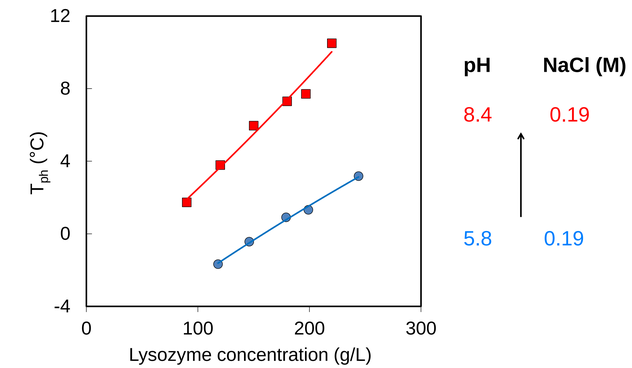


Phase-contrast light microscopy images showing that globular (protein-rich) particles of lysozyme were stable at room temperature in HEPES buffer solutions. These globular particles cannot be seen in Phosphate or Tris buffer at room temperature due to LLPS reversibility.

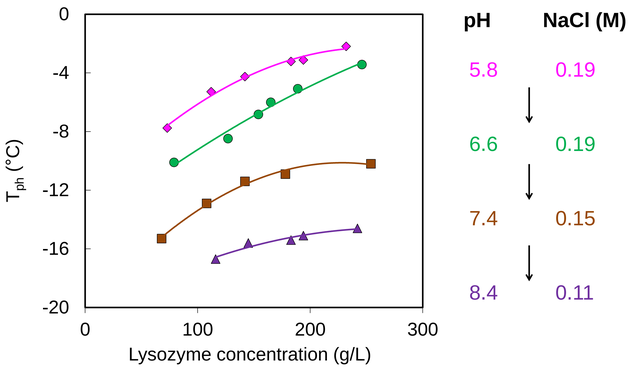
2. Effect of pH on LLPS of Lysozyme in the presence of HEPES

Our LLPS studies on lysozyme solutions in citrate buffer (pH 5.8) and Tris buffer (pH 8.4) showed that LLPS temperature increase with pH, consistent with literature. On the other hand, our LLPS studies in HEPES buffer showed the opposite behavior.

LLPS phase boundary in Tris (■) and Citrate (●) buffers



LLPS in HEPES buffer at four pH values



No LLPS is observed in the presence of HEPES 0.4 M and in the absence of NaCl. This indicates that NaCl not HEPS promotes LLPS.

Proposed Mechanism

HEPES is responsible for a physical cross-linking between lysozyme proteins. The formation of protein-rich droplets enhances this cross-linking mechanism, thereby producing globular protein aggregates that remain stable at high temperatures.

Future Directions

- Identify molecular interactions responsible for this protein cross-linking.
- Characterize stability of these protein globular condensates above LLPS boundary.

Conclusion

LLPS of protein solutions may strongly depend on the chemical nature of buffer.

Acknowledgement

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