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Filamentous Chaperone Protein-Based Hydrogel Stabilizes Enzymes against Thermal Inactivation

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Materials and Methods

Protein Expression and Purification

The genes encoding γPFD, γPFD-K_2E_2 and OPH were synthesized as gBlocks gene fragments (Integrated DNA Technologies) and were inserted into the multiple cloning sites of the pET- 19b plasmid (Novagen) using the Gibson assembly (New England Biolabs). The assembled plasmids were transformed into T7 Express competent cells, which were grown at 37°C in LB Broth (Lennox) containing 100 μg/mL ampicillin up to OD_{600} = 0.6. γPFD, γPFD-K_2E_2 protein expression was induced by adding IPTG to final concentration of 0.5 mM and cells were grown for an additional 16 hours at 25°C. The cells were harvested by centrifugation at 5000 rpm for 20 minutes, lysed by French press, and centrifuged at 13,000 rpm for 60 minutes. For γPFD, γPFD-K_2E_2 purification, the resulting supernatant was then purified by multimodal chromatography resin (Capto Core 700, GE healthcare) using AKTA FPLC. The proteins were loaded on the column with the equilibrium buffer (100 mM NaCl, 50mM Tris, pH 7.4). The elution peak was inspected using SDS-PAGE and SimplyBlue staining (Invitrogen), and the pure target protein was dialyzed overnight against water. Finally, the purified proteins were flash-frozen with liquid N_2 and lyophilized for storage at -80°C.

Expression of the OPH, T7 Express competent cells was induced with 0.5 mM IPTG for 16 h at 30 °C before being incubated in the presence of 1 mM $CoCl_2$ for the last 5 hours. The cells were harvested by centrifugation at 5000 rpm for 20 minutes, lysed by French press, and centrifuged at 13,000 rpm for 60 minutes. After centrifugation the supernatant was purified on an AKTA FPLC with HisTrap prepacked column (GE healthcare). The elution peak was inspected using SDS- PAGE

and SimplyBlue staining (Invitrogen). Finally, the purified proteins were flash-frozen with liquid N_2 and lyophilized for storage at -80°C.

TEM Imaging

γPFD, and γPFD-K_2E_2 were imaged by transmission electron microscopy using a Tecnai 12 120KV (FEI Company) and images were captured by a Gatan Ultrascan 1000 digital micrograph. The TEM samples were prepared by depositing 0.5 mg/ml of protein onto 400 mesh carbon/formvar coated copper grids (Electron Microscopy Sciences) for 2 min. Then the TEM sample was washed with water and subsequently stained with 2 % uranyl acetate solution for 1 min.

Hydrogel Formation and Disassembly

Lyophilized protein powder was dissolved in Tris buffer (100 mM NaCl, 50mM Tris, pH 8.0) to the desired final concentration. Lyophilized protein powder was allowed to dissolve and cure at 4°C for 12 hours.

γPFD-K_2E_2 hydrogel (100 µL) was formed in Tris buffer (100 mM NaCl, 50mM Tris, pH 8.0) at 2%wt. Then 10uL of 5M KCl was added to the gel. The mixture was fully vortexed and left still for 10 minutes before inverting and photographing for Figure S6.

SEM Imaging

Scanning electron microscopy (FEI Quanta 3D FEG, Thermo Fisher Scientific) was used to observe the morphology of the lyophilized protein samples. The samples were directly deposited on conductive carbon tape, and coated with Au and then observed by SEM.

Rheology Tests

Oscillatory shear rheology tests were performed with a Physica MCR 301 parallel plate rheometer (Anton Paar), using an 8-mm diameter top plate. 50 µL of protein sample was formed in situ between the parallel plates at a gap width of 0.8 mm and were allowed to cure in the humidified chamber. Amplitude sweep tests were performed at 1 Hz frequency. For all hydrogel samples tested in this study, 1% applied strain was within the linear viscoelastic range.

Entrapped Enzyme Release and Hydrogel Erosion

Cytochrome C, HRP, hemoglobin, catalase (Sigma-Aldrich) individually dissolved in Tris buffer were mixed with hydrogel. Each mixture was incubated at 4 °C for 16 hours. 200 µL Tris buffer was added to the enzyme-hydrogel mixture to initiate the passive diffusion of enzyme from the hydrogel. A small aliquot of buffer was taken at respective time points to measure the heme UV absorbance from the released enzyme. The percentage of enzyme released was calculated by dividing released enzyme UV absorbance by the fully released enzyme UV absorbance.

50-uL hydrogels were formed and cured as described above. 200 µL Tris buffer was added and incubated at room temperature. At each time point of measurement, a small aliquot of buffer was removed for the UV absorbance measurement. The percentage of protein released was calculated by dividing released hydrogel protein UV absorbance at 275 nm by the fully dissolved gel UV absorbance. The gel was fully dissolved by adding excess water (2:1 v/v) and vortexing.

Enzyme Protection Assay

All samples were prepared in Tris buffer. To all sample tubes, equal volume of Tris buffer, 2%wt γPFD and γPFD-K_2E_2 were mixed with equal volume of enzyme solution. For HRP the final enzyme concentration was 0.05 µM; for OPH it was 0.1mg/ml. All sample tubes were then vortexed and incubated at 25°C for 1 hr before heat treatment. After heat treatment in a BioCision CoolRack XT PCR96 module in a preheated oven at 80°C and 25°C for 30 min, the heated and control sample were cooled to 25°C for 5 min before enzyme activity assays. For HRP, 2 µL of sample was mixed with 100 µL reaction solution with 0.01mg/ml 3,3',5,5'-tetramethylbenzidine (TMB) and 0.003% H_2O_2. Reactions were stopped with 100 µL of 2M HCl after 5 min and absorbance at 450 nm was measured. The product concentration was calculated using an extinction coefficient of ε_{450nm} = 59,000 $M^{-1} \cdot cm^{-1}$ for 3,3',5,5'-tetramethylbenzidine diimine[1]. For OPH, following similar heat treatment at 60°C or 25°C for 30 min, the heated and control samples were cooled to 25°C for 5 min, after which 2 µL of sample was mixed with 100 µL reaction solution containing 1 mM parathion methyl and 100 µM $CoCl_2$, and absorbance at 410 nm was measured. Reaction rates were constant for up to 5 minutes at each enzyme concentration used, as determined by making absorbance measurements at multiple times (data not shown). An extinction coefficient of ε_{410nm} = 16,500 $M^{-1} \cdot cm^{-1}$ was used to calculate the concentration of *p*-nitrophenol[2].

γPFD:
MVNEVIDINEAVRAYIAQIEGLRAEIGRLDATIATLRQSLATLKSLKTLGEGKTVLVPVGSIAQV EMKVEKMDKVVVSVGQNISAELEYEEALKYIEDEIKKLLTFRLVLEQAIAELYAKIEDLIAEAQQ TSEEEKAEEEENEEKAE
γPFD-K_2E_2:
MVNEVIDINEAVRAYIAQIEGLRAEIGRLDATIATLRQSLATLKSLKTLGEGKTVLVPVGSIAQV EMKVEKMDKVVVSVGQNISAELEYEEALKYIEDEIKKLLTFRLVLEQAIAELYAKIEDLIAEAQQ TSEEEKAEEEENEEKAEKVSALKEKVSALKEVSALEKEVSALEKE
OPH:
MGHHHHHHSIGTGDRINTVRGPITISEAGFTLTHEHICGSSAGFLRAWPEFFGSRKALAEKA VRGLRRARAAGVRTIVDVSTFDIGRDVSLLAEVSRAADVHIVAATGLWFDPPLSMRLRSVEE LTQFFLREIQYGIEDTGIRAGIIKVATTGKATPFQELVLKAAARASLATGVPVTTHTAASQRDGE QQAAIFESEGLSPSRVCIGHSDDTDDLSYLTALAARGYLIGLDHIPHSAIGLEDNASASALLGIR SWQTRALLIKALIDQGYMKQILVSNDWLFGFSSYVTNIMDVMDRVNPDGMAFIPLRVIPFL REKGVPQETLAGITVTNPARFLSPTLRAS

Table S1. Amino acid sequences of the recombinantly expressed proteins used in this study.

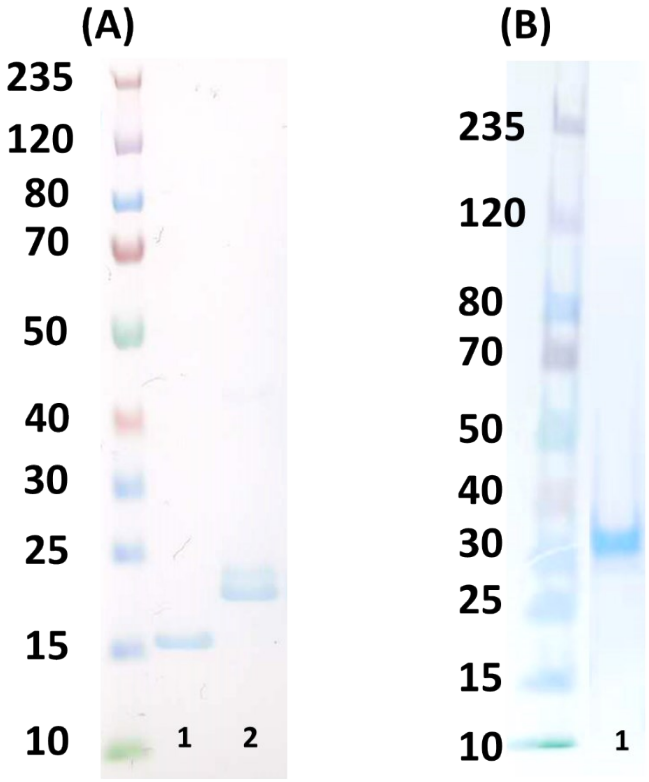

Figure S1. SDS-PAGE results showing the γPFD(16kDa), γPFD-K_2E_2 (19kDa) and OPH (37kDa). Loaded samples were (A) γPFD (lane 1), γPFD-K_2E_2 (lane 2), and (B) OPH (lane 1).

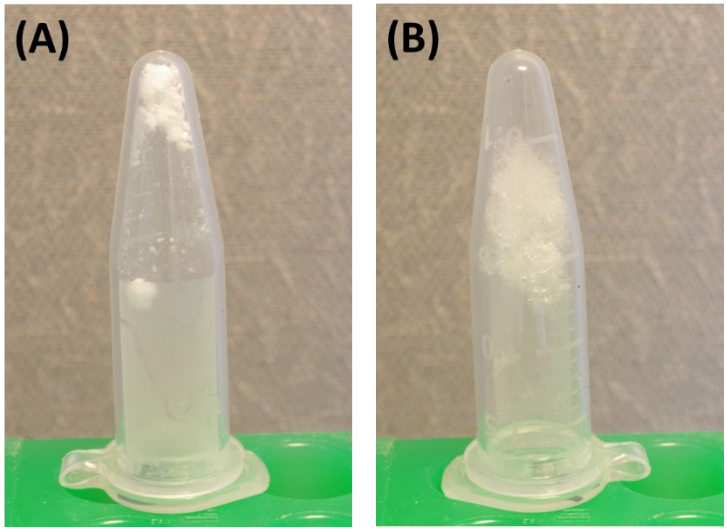

Figure S2. Lyophilized γPFD and γPFD-K_2E_2 hydrogel after liquid N_2 flash freezing. (A) γPFD; (B) γPFD-K_2E_2 hydrogel.

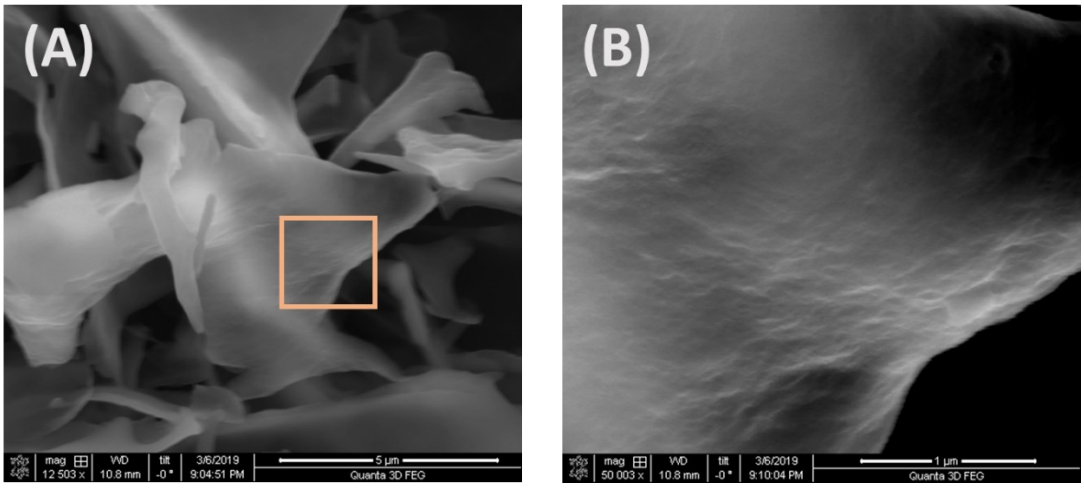

Figure S3. SEM images of lyophilized prefoldin. (A) Images at 12503× magnification and (B) at 50003× magnification.

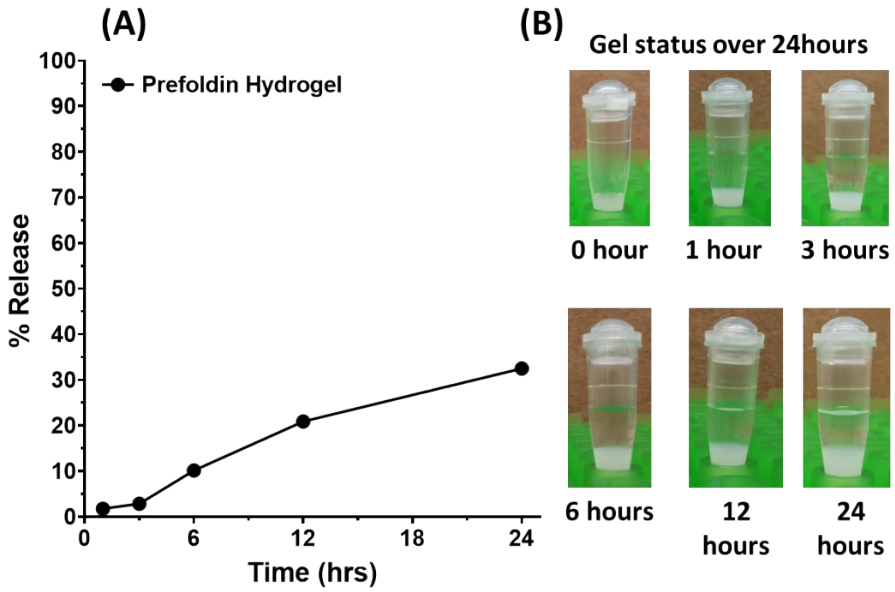

Figure S4. (A)Total protein release profile of the 5 wt% γPFD-K_2E_2 hydrogels over 24 hours (n=3). (B) Hydrogel status over time in a microcentrifuge tube containing 50 µL of gel in 100 µL of Tris buffer; the gel does not fully dissolve after 24 hr.

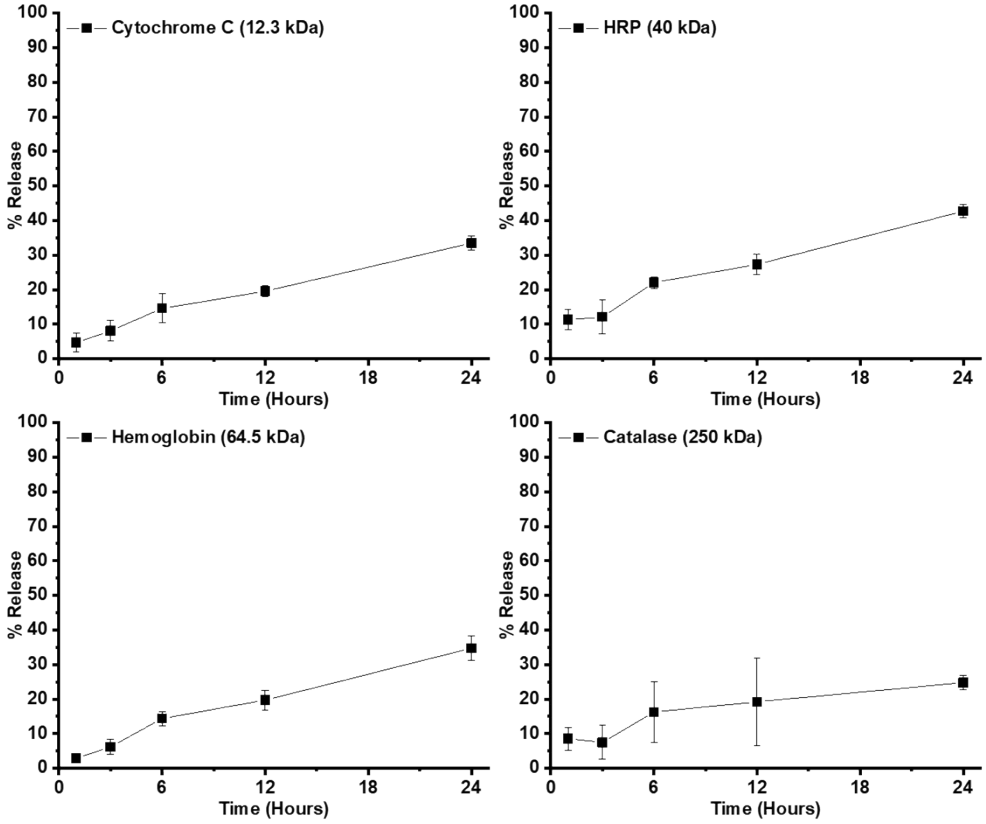

Figure S5. Release profiles of entrapped enzymes of various molecular weights from γPFD-K_2E_2 hydrogel over 24 hr (n=3).

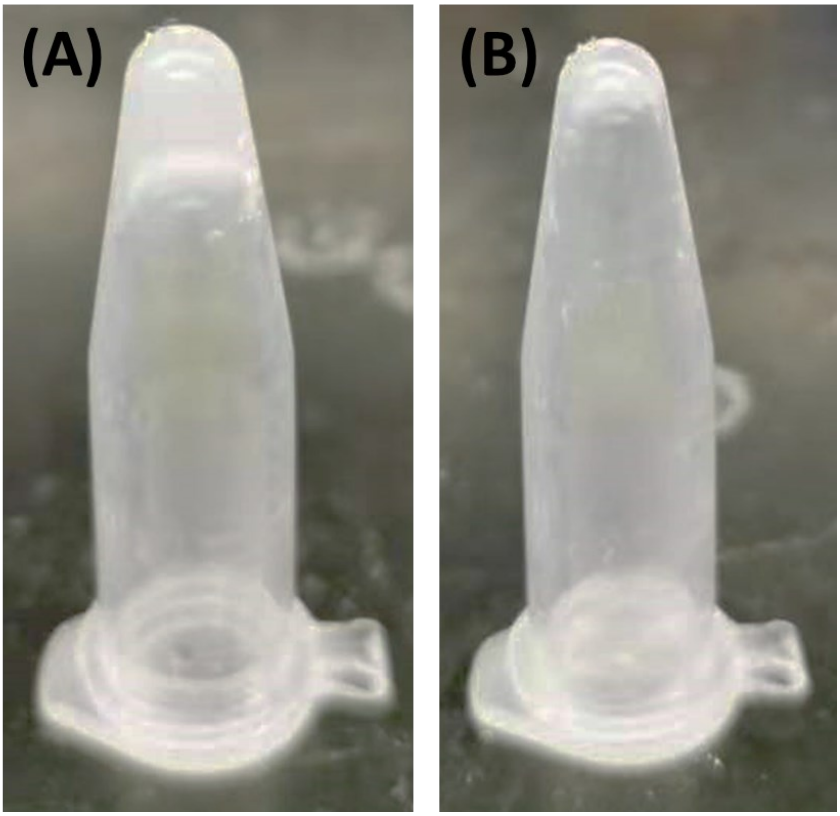

Figure S6. Salt effect on gelation of γPFD-K_2E_2 hydrogel. γPFD-K_2E_2 hydrogel before (A) and after (B) incubating for 10 min in 500 mM KCl.

References

1. V. Čunderlová, A. Hlaváček, V. Horňáková, M. Peterek, D. Němeček, A. Hampl, L. Eyer and P. Skládal, *Microchimica. Acta.*, 2016, **183**, 651-658.

2. C. DelRe, C. Huang, T. Li, P. Dennis, E. Drockenmuller and T. Xu, *ACS Appl. Mater. Interfaces*, 2018, **10**, 44216-44220.