Electronic Supplementary Information

Designing biological fluid inspired molecularly crowded ionic liquid media as sustainable packaging platform for Cytochrome c

Kavya Bhakuni,[a] Meena Bisht,[ab] Pannuru Venkatesu,*[a] Dibyendu Mondal*[c]

[a]Department of Chemistry, University of Delhi, Delhi – 110 007, India.

[b]Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago 3810−193 Aveiro, Portugal

[c]Centre for Nano & Material Sciences, JAIN (deemed to be University), Jain Global Campus, Bangalore-562112, India.

Experimental details

Materials. Cytochrome c from the equine heart (≥95%), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (>98%), hydrogen peroxide solution (H_2O_2) 30% (w/w) in H_2O, ficoll 70 (F70), absolute ethanol (≥99.8%) were purchased from Sigma-Aldrich. PEG 12 kDa (P12) was purchased from Fluka Analyticals. Highly pure anhydrous sodium phosphate monobasic, sodium phosphate dibasic dehydrate and sucrose (Suc) were purchased from Sisco Research Lab (SRL). Choline dihydrogen phosphate (Cho-Dhp) was purchased from IoliTec. All the solutions were prepared in sodium phosphate buffer solution (10 mM) of pH 7 using deionized water.

Designing bio-fluid inspired media. To design molecularly crowded system different amalgamations of model crowding agents (P12, F70, and Suc) with Cho-Dhp were utilized. The bio-fluid inspired media was prepared by using a combination of Cho-Dhp (0.5 g/mL) with one, two and three crowding agents and the total concentration of crowders were 50 mg/mL in phosphate buffer of pH 7. The mixture that was prepared was thoroughly mixed in the vortex. The pH of the final solution obtained was adjusted to pH 7 using 0.1 M NaOH or 0.1 M HCl solutions.

Activity measurements. Cyt c exhibits peroxidase activity, which was analyzed using ABTS as a substrate in presence of H_2O_2. Cyt c carry out oxidation of ABTS resulting into green colored $ABTS^+$ radical, this reaction was initiated by adding H_2O_2. The changes in absorption at 420 nm was observed with time for five minutes to monitor the generation of $ABTS^+$. All activity measurements were carried out in solution,

where Cyt c concentration was 2 µM, ABTS was 3 mM, H_2O_2 was 1 mM, Cho-Dhp was 0.5 g/mL and concentration of different crowders was 50 mg/mL.

For the time-dependent study, the respective enzyme solutions in crowded media were incubated at room temperature for 30 days. The remaining activity was calculated by considering the difference of the initial activity and the activity after the 30 days. The effect of temperature on peroxidase activity of Cyt c in crowder-IL media was determined after 45 mins incubation at 20, 40, 60, 80 and 100 °C. The percentage relative activity in all cases was calculated by considering 100 % enzymatic activity in the presence of sodium phosphate buffer at pH 7.

The concentration of the product ($ABTS^{+\bullet}$) was calculated according to the Beer–Lambert Law using the molar extinction coefficient of $ABTS^{+\bullet}$ at 415 nm (ε_{415}=3.6 × 104 $M^{-1}cm^{-1}$).[1] One unit of activity is defined as the amount of product that will be produced by 1 µmol of cyt c per min under the reaction.

Proteolytic activity of Stem Bromelain (SB). Activity of was essayed using UV–vis spectrophotometer, keeping concentration of SB as 0.5 mg/ml. Samples were prepared as described earlier. Denatured casein solution (250 µL, 2.0% w/v) was mixed with 250 µL sample, followed by 10 min of incubation at 37 °C. To stop the reaction, 500 µL of ice-cold TCA (110 mM) was added immediately. Samples were then centrifuged followed by absorption measurement of the supernatant solution at 275 nm. Activity (Units/mL) of SB was determined using following equation:

$$Activity = \frac{\mu mole\ of\ tyrosine\ equivalent\ released\ \times V}{time\ of\ assay\ (mins) \times v_1 \times v_2}$$

Where, v_1 and v_2 is the volume of the enzyme (mL) and volume of sample (mL) respectively. The total volume of the assay (mL) is V.

Here, one unit of activity is defined as the quantity of enzyme that converts 1µmole of substrate per mL. The data presented here is obtained after respective blank subtraction.

Spectroscopic studies. The structural stability of Cyt c in presence of crowders and IL was analyzed by UV-visible spectroscopy and Circular dichroism (CD) spectroscopy. The enzyme concentration was 0.5 mg/mL in 10 mM phosphate buffer (pH 7) while concentration of Cho-Dhp and crowders (P12, Suc, F70) were 0.5 g/mL and 50 mg/mL respectively. The equilibration time for all the samples was 45 mins. All the spectra presented here are obtained after subtracting respective blank solutions.

Differential Scanning Calorimetric (DSC) studies- The thermal stability of the Cyt c was assessed in presence of Cho-Dhp (0.5 g/mL), crowders (50 mg/mL) and its combination with the help of nano-DSC (TA Instruments, New Castle, DE, USA). Because of heat capacity limitation, direct analysis of the samples was not possible. Enzyme Cyt c was precipitated from crowded-IL media using cold ethanol and again dissolved in phosphate buffer. All samples were degassed for 5 mins at 25 °C prior to scans. Temperature range for the study was 30 – 120 °C, heating rate of 2 °C/min and 600 sec of equilibration time. During heating, cells were pressurized to 3 atmospheres. Data were analyzed using the DSC Nanoanalyze software and T_m was calculated. The buffer baseline was subtracted from the data reported here.

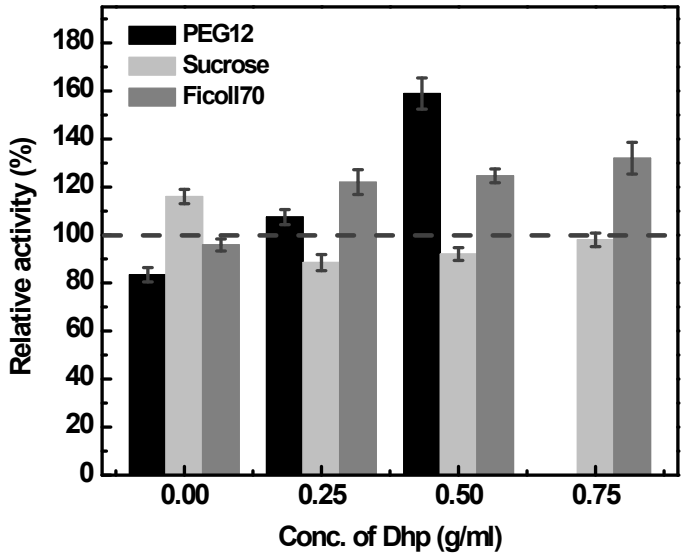

Figure S1. Relative activity of Cyt c at various concentration Cho-Dhp in aqueous solution and crowders (50 mg/mL). As mixture of PEG12 (50 mg/mL) and Dhp (0.75g/mL) was not homogeneous the data is not reported here.

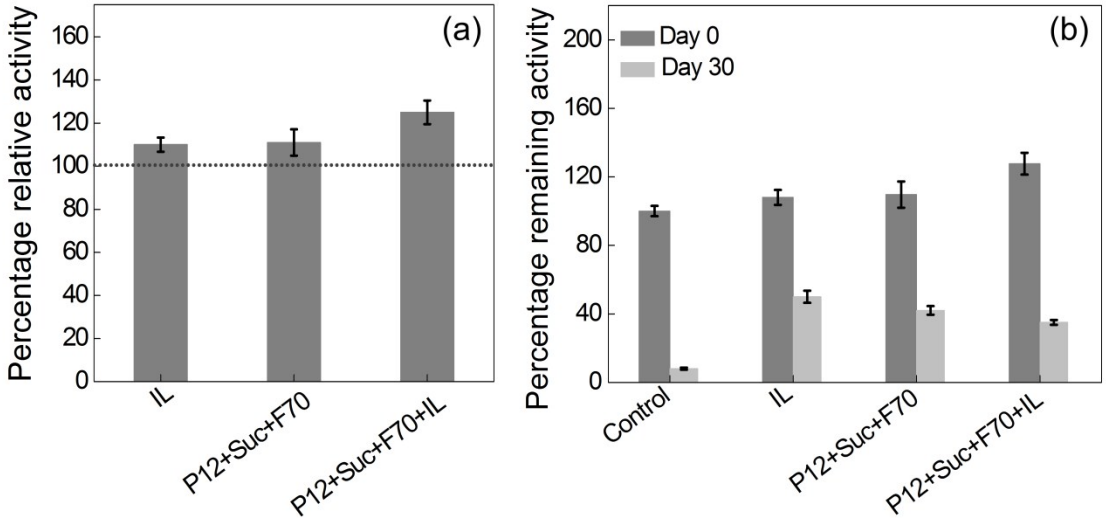

Figure S2 (a) Percentage relative activity of Cyt c in existence of mixture of all three crowders (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL), in phosphate buffer pH 7. **(b)** Time dependent study of activity of Cyt C after incubation for one-month at room temperature in presence of mixture of all three crowders (50 mg/mL) with and without Cho-Dhp (0.5 g/mL), followed by dissolution in phosphate buffer pH 7.

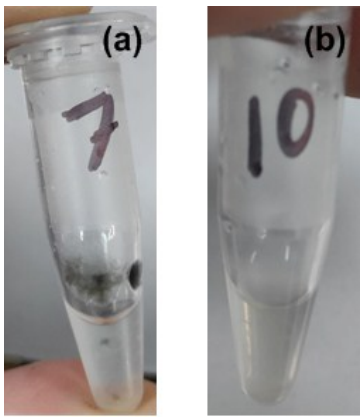

Figure S3. Images of Cyt c in (a) Suc + F70 and (b) Suc + F70 + IL in phosphate buffer at pH 7, after 30 days at room temperature.

Table -S1 Activity of Cyt c in presence of different crowders and their mixture (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7.

Sample Name	Activity (unit/µmol.E)
Control	35.0
IL	38.5
P12	30.8
Suc	45.5
F70	40.6
P12+IL	42.3
Suc+IL	32.2
F70+IL	43.4
P12+suc	31.5
Suc+F70	36.4
F70+P12	36.7
P12+suc+IL	36.0
Suc+F70+IL	56.1
F70+P12+IL	53.5
P12+Suc+F70	38.8
P12+Suc+F70+IL	43.7

Table -S2 Time-dependent study of activity of Cyt C after incubation for one month at room temperature in presence of crowders and their mixture (50 mg/mL) with and without Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7.

Sample Name	Activity (unit/µmol.E) on 1st day	Activity (unit/µmol.E) on 30^{th} day
Control	35	2.8
IL	37.8	17.5
P12	31.5	10.5
Suc	43.7	28.0
F70	39.9	19.2
P12+IL	43.7	19.6
Suc+IL	34.3	24.5
F70+IL	45.5	17.5
P12+suc	31.3	17.5
F70+P12	37.1	10.5
P12+suc+IL	38.6	11.2
Suc+F70+IL	56.3	13.3
F70+P12+IL	52.9	19.6
P12+Suc+F70	38.3	14.7
P12+Suc+F70+DHP	44.6	12.2

Table-S3 Activity (unit/µmol.E) of Cyt c as a function of temperature in different crowders and their mixture (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7.

Sample Name	20 ^{O}C	40 ^{O}C	60 ^{O}C	80 ^{O}C	100 ^{O}C
Control	35.0	37.6	41.0	43.1	41.3
IL	38.5	40.3	52.5	95.7	80.1
P12	30.1	41.5	63.1	73.5	72.1
Suc	42.2	70.1	84.2	91.9	51.3
F70	41.3	68.2	79.8	87.5	91.2
P12+IL	43.4	50.3	92.8	103.2	68.6
Suc+IL	33.3	52.5	70.3	91.2	84.1
F70+IL	43.1	77.0	105.0	128.9	126
P12+Suc	32.5	39.9	63.8	82.7	57.6
Suc+F70	39.2	59.5	66.5	84.2	80.5
F70+P12	37.8	42.4	61.3	78.2	77.0
P12+Suc+IL	27.3	54.5	85.7	104.3	71.4
Suc+F70+IL	45.5	64.75	98	105	104.3
F70+P12+IL	49	58.2624	94.5	88.36138	86.8

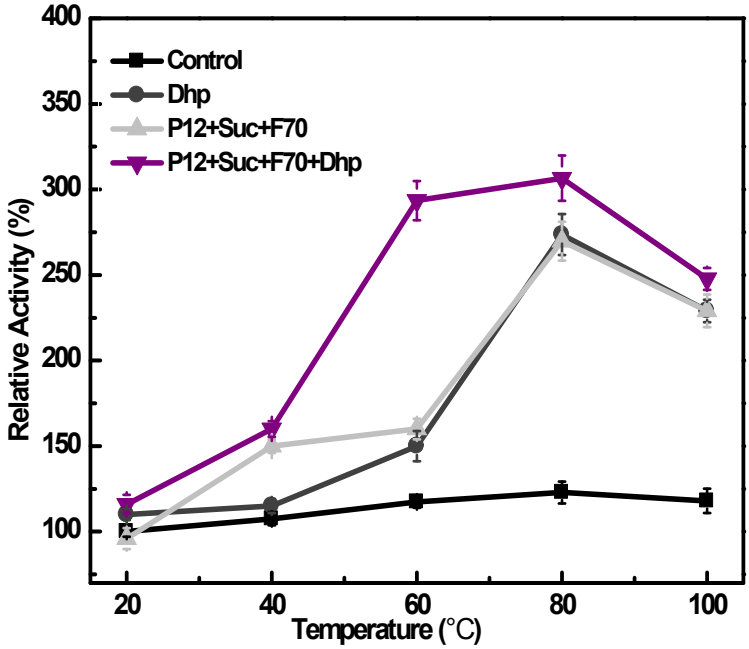

Figure S4. Percentage relative activity of Cyt c as a function of temperature in existence of mixture of all three crowding agents (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7

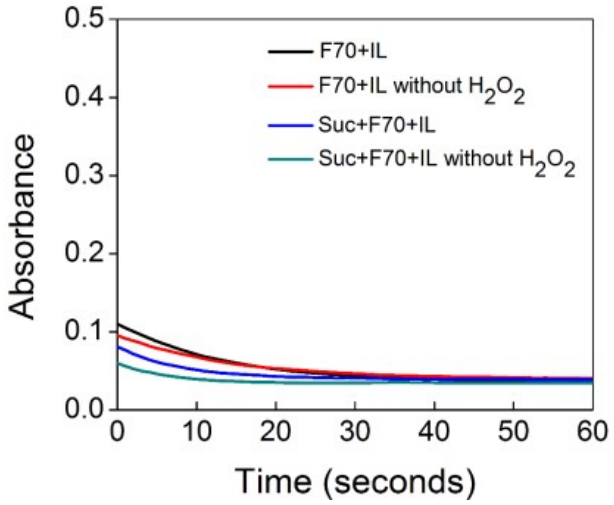

Figure S5. Background interference of molecularly crowded IL media prepared in phosphate buffer of pH 7 measured at 100 °C.

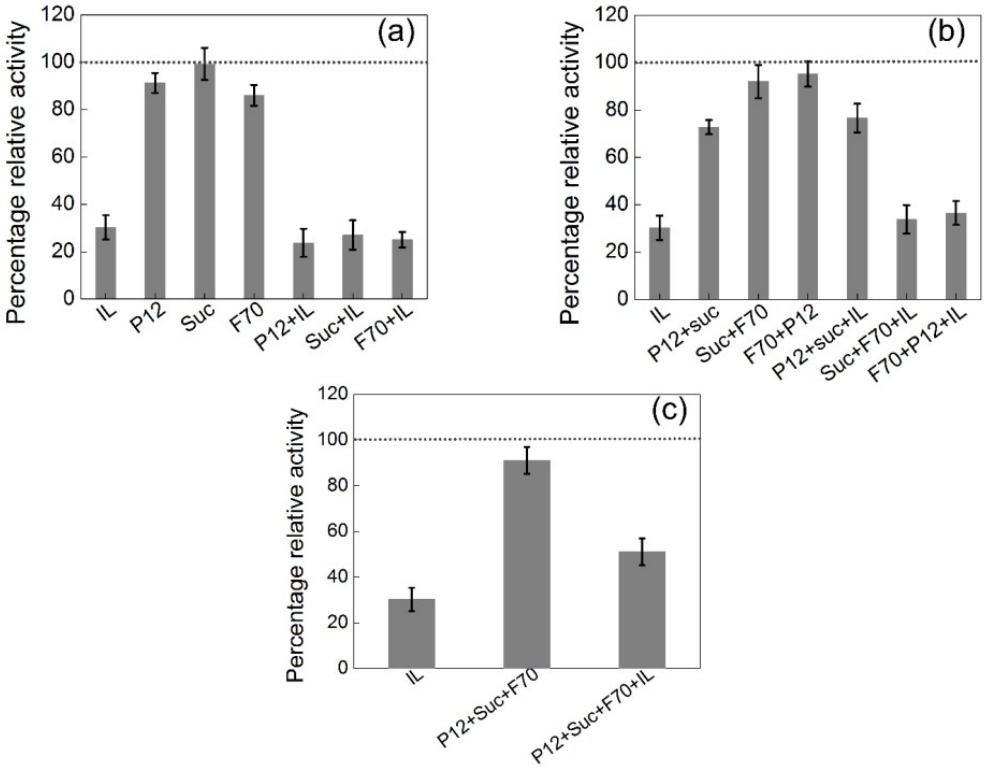

Figure S6. Percentage relative proteolytic activity of SB in existence of (a) different crowders (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL) (b) mixture of two crowders (50 mg/mL) in presence and absence of Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7. (c) mixture of three crowders (50 mg/mL) with and without Cho-Dhp (0.5 g/mL) followed by dissolution in phosphate buffer pH 7 using denatured casein as substrate.

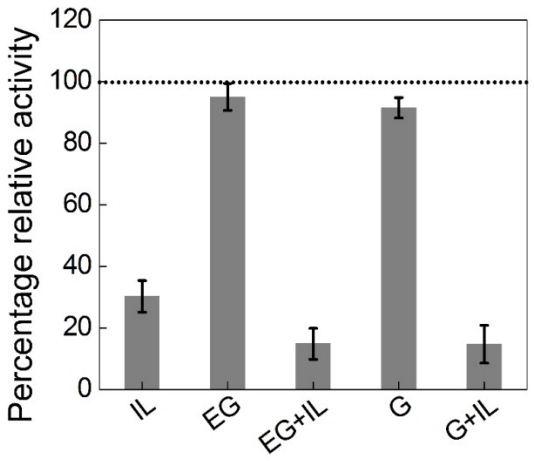

Figure S7. Percentage relative proteolytic activity of SB in existence of Ethylene glycol (EG) (50 mg/mL) in absence and presence of Cho-Dhp (0.5 g/mL) and glycerol (G) (50 mg/ml) in presence and absence of Cho-Dhp (0.5 g/mL) in phosphate buffer pH 7 using denatured casein as substrate. The dashed line depicts relative activity of native SB which was assumed as 100 %.

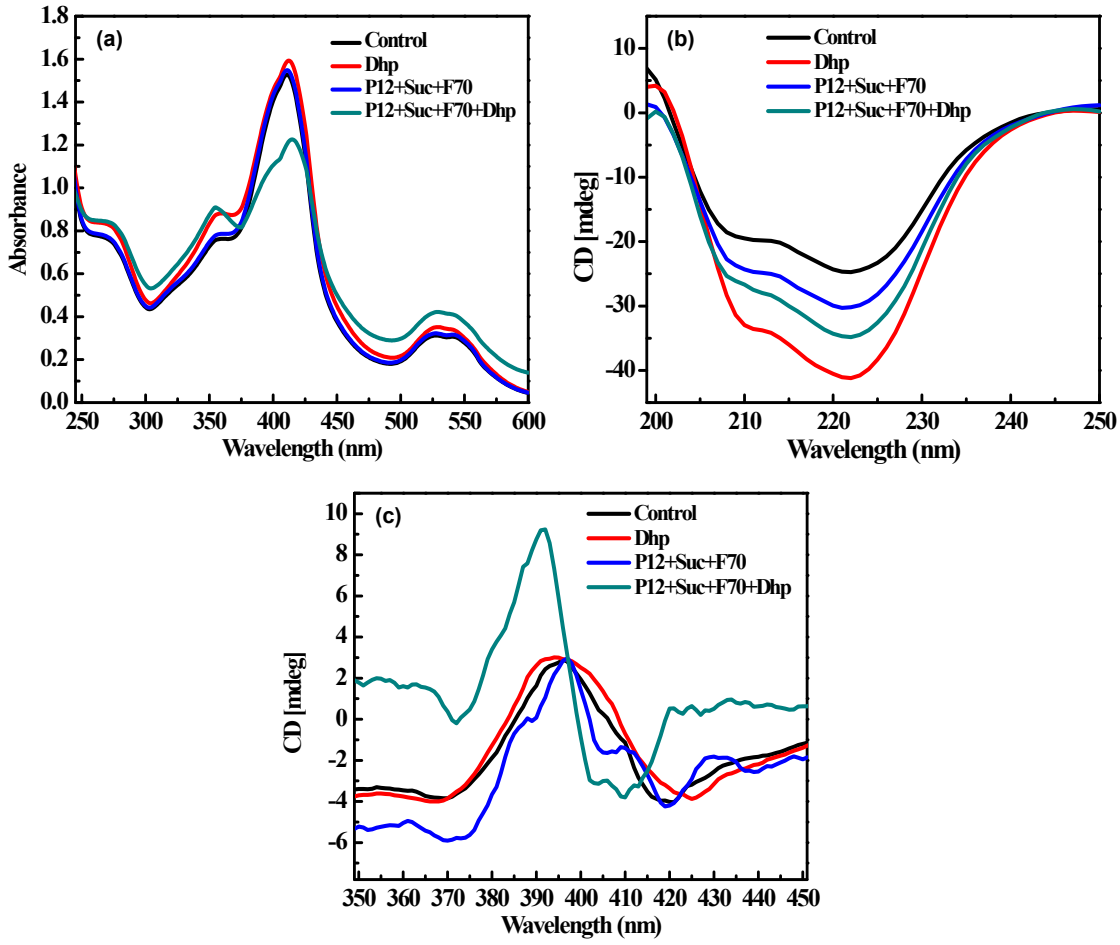

Figure S8. (a) UV-visible spectra, (b) far UV-CD spectra and (c) Soret band spectra of Cyt c in mixture of all three crowding agents in presence and absence of Dhp in phosphate buffer of pH 7.

Molecular docking studies. A molecular docking simulation for Cyt c in presence of crowding agents and IL was performed using MVD trail version (v 6.0) downloaded from Molegro with the default parameter settings.[2] For the docking purpose, we utilized the theoretical model of Cyt c from the PDB bank (PDB code: 1HRC) and energy was minimized using online software Yasara. Molecular structures of IL Cho-Dhp as well as crowding agents (PEG, Sucrose and Ficoll) were prepared using Chem Draw and energy was optimized using Hyper Chem 8.0 with the default settings. We used repeating units (10 monomer) PEG and ficoll due to limitations of the software and system. [2, 3] The PDB file of Cyt c and optimized MOL file of crowding agents and Cho-DHP was imported into the docking program. The binding sites or cavities were automatically detected by the software (restricted within spheres of radius 15 Å). The other

parameters were grid resolution (0.3 Å), maximum population size (50), maximum iteration (1500 Å), energy threshold (100) and maximum steps (300). The number of runs was fixed to be 100. Docking results were scrutinized based on the rerank score generated by the software. Out of all the possible poses attained only the one with the highest rerank score are presented here.

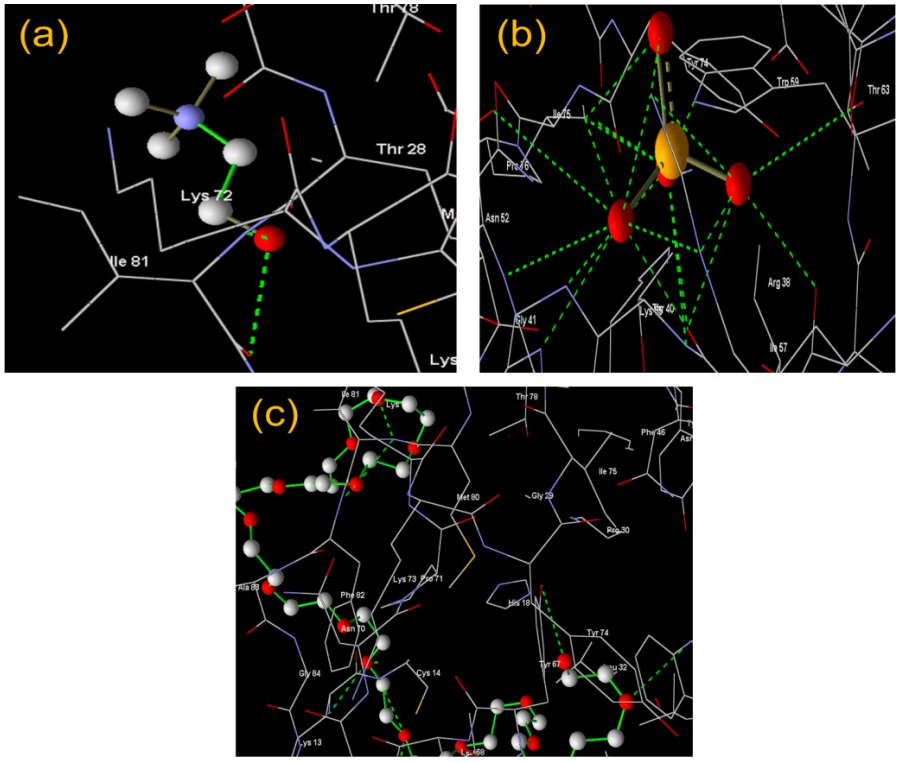

Figure S9. The Hydrogen bond interactions (green dashed lines) predicted by docking analysis in 44 Å cavities on Cyt c surface in presence of all three additives (a) Choline (b) dihydrogenphosphate (c) PEG molecule. The Cyt c exhibits 2 H-bond with Cho, 20 H-bond with Dhp and 12 H-bond with PEG molecules.

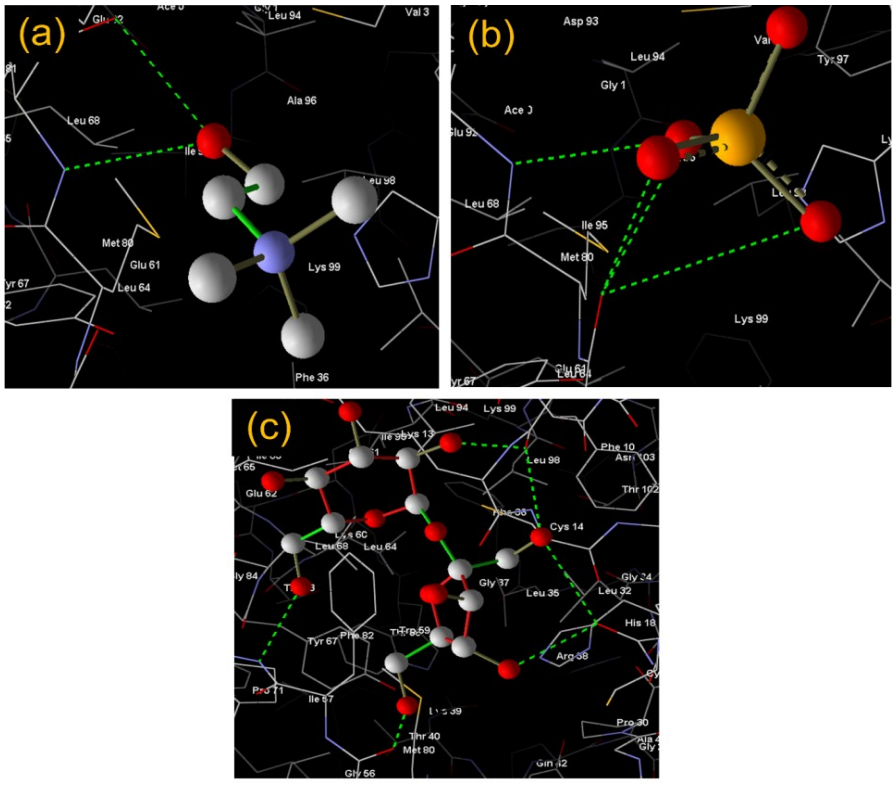

Figure S10. The Hydrogen bond interactions (green dashed lines) predicted by docking analysis in 44 Å cavity on Cyt c surface in presence of all three additives (a) Choline (b) dihydrogenphosphate (c) sucrose molecule. Cyt c displays 2 H-bond with Cho, 5 H-bond interactions with Dhp and 7 H-bond interactions with Sucrose.

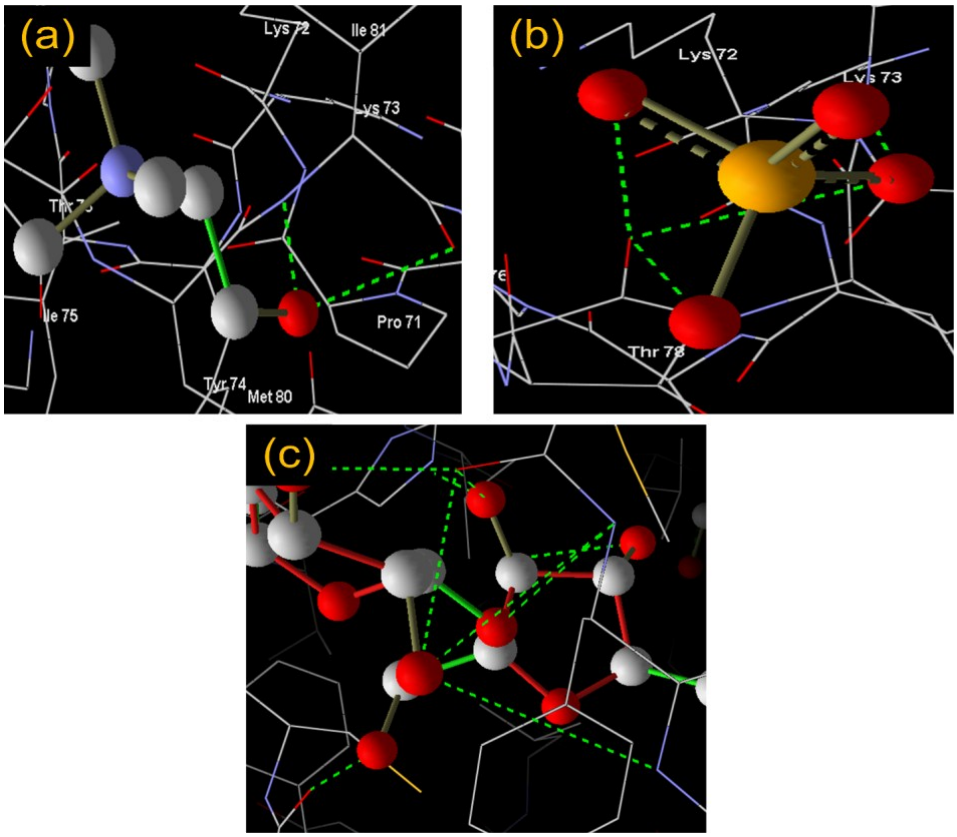

Figure S11. The Hydrogen bond interactions (green dashed lines) predicted by docking analysis in 44 Å cavity on Cyt c surface in presence of all three additives (a) Choline (b) dihydrogenphosphate (c) Ficoll molecule. Ficoll, shows 136 H-bond interactions with Cyt c in addition to 2 H-bond with cho and 2 H-bond with Dhp.

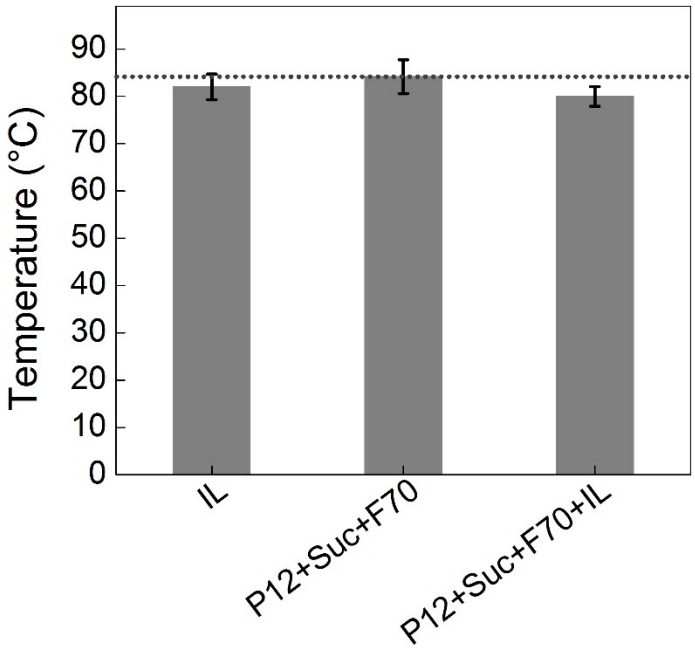

Figure S12. The T_m (°C) value obtained from DSC thermograms of Cyt c after back extraction from solution containing mixture of all three crowders (50 mg/mL) with and without Cho-Dhp (IL) (0.5 g/mL), followed by dissolution in phosphate buffer pH 7.

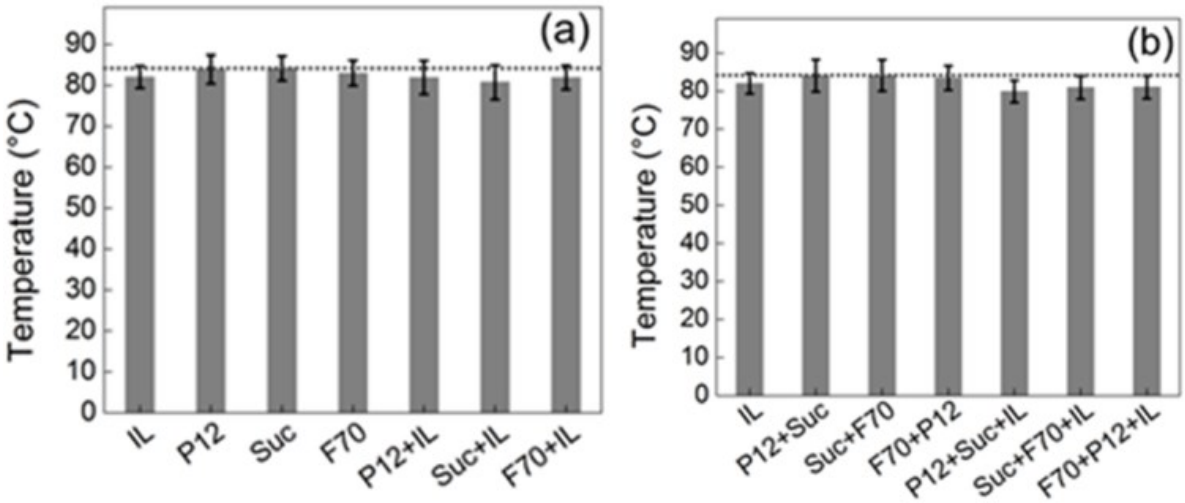

Figure S13. The Tm (°C) value obtained from DSC thermograms of Cyt c after back extraction from solution containing (a) crowders (50 mg/mL) with and without Cho-Dhp (0.5 g/mL) (b) mixture of two crowders (50 mg/mL) with and without Cho-Dhp (0.5 g/mL) followed by dissolution in phosphate buffer pH 7.

Table S4: Thermodynamic parameter predicted from DSC analysis.

S.no.	Sample	T_m (K)	$\Delta_u H$ (T_m)(KJ/mol)	$\Delta_u S$ (T_m)(KJ/mol/K)
1	Control	357.15	491.7	1.37
2	IL	355.14	339.2	0.9622
3	P12	357.01	397	1.1077
4	Suc	357.27	433.6	1.212
5	F70	356.1	383.4	1.073
6	P12+IL	355.03	364.9	1.027
7	Suc+IL	353.86	371.3	1.053
8	F70+IL	355.03	368.4	1.037
9	P12+Suc	357.16	340.3	0.952
10	Suc+F70	357.24	346	0.966
11	F70+P12	356.55	409.2	1.1446
12	P12+Suc+IL	353.02	406.3	1.1529
13	Suc+F70+IL	354.04	216.5	0.617
14	F70+P12+IL	354.13	403.8	1.138
15	P12+Suc+F70	357.27	440.8	1.23
16	P12+Suc+F70+IL	353.1	447.2	1.262

References:

1 N. H. Kim, M. S. Jeong, S. Y. Choi, and J. H. Kang, Bull. Korean Chem. Soc. 2004, 25,1989-1992.

2. K. Bhakuni, P. Venkatesu, Crowded milieu tuning the stability and activity of stem bromelain, Int. J. Biol. Macromol. 109 (2018) 114–123.

3. M. Shakir, M. S. Khan, U. Baig, M. F. Alam, H. Younus, M. Alam, In vivo cytotoxicity, molecular docking and study of yeast alcoholdehydrogenase on polycarbazole-titanium dioxide nanocomposite, J. Mol. Catal. B. Enzym. 134 (2016) 79-88.