Supporting information

Improving the Activity of Horseradish Peroxidase in Betaine-based Natural Deep Eutectic Systems

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Supplementary Tables

Table S1. Number of molecules in each simulation box.

System	*N° W*	*N° B*	*N° G*	*N° Xyl*	*N° Treh*	*N° Sorb*	*N° Suc*	*N° Pro*
Water	23000	-	-	-	-	-	-	-
BXylW	23000	398	-	199	-	-	-	-
BTrehGlyW	23000	214	321	-	107	-	-	-
BSorbW	23000	282	-	-	-	282	-	-
BSucProW	23000	260	-	-	-	-	104	104
100 wt% BGly	23000	343	686	-	-	-	-	-
95 wt% BGly	23000	323	646	-	-	-	-	-
90 wt% BGly	23000	302	604	-	-	-	-	-
85 wt% BGly	23000	282	564	-	-	-	-	-
80 wt% BGly	23000	262	524	-	-	-	-	-

*W: water, B: betaine, G: glycerol, Xyl: D-(+)-xylose, Treh: trehalose, Sorb: D-sorbitol, Suc: sucrose, Pro: DL-proline.

Table S2 – Secondary structure contents (α-helix, β-strand, turns and random coils) of HRP after incubation on PBS and NADES at 37 ºC (for 24 h), 60 ºC (for 24 h) and 80 ºC (for 4 h). Control experiments for each system were determined without incubation (t=0 h).

		α-helix	**β-strand**	**Turns**	**Random Coils**
PBS	*Control*	31	9	16	44
	37 ºC	29	17	15	39
	60 ºC	20	20	15	45
	80 ºC	22	15	16	47
BXylW	*Control*	20	23	15	42
	37 ºC	17	26	15	43
	60 ºC	10	26	15	49
BTrehGlyW	*Control*	26	18	15	41
	37 ºC	26	13	17	45
	60 ºC	28	10	16	46
	80 ºC	28	7	17	49
BSorbW	*Control*	35	5	17	43
	37 ºC	32	10	16	42
	60 ºC	35	6	17	43
	80 ºC	21	22	15	42
BSucProW	*Control*	28	20	14	38
	37 ºC	21	23	16	40
	60 ºC	25	21	15	40
	80 ºC	27	20	15	38
BGly	*Control*	24	17	16	43
	37 ºC	33	9	16	43
	60 ºC	30	11	16	44
	80 ºC	31	5	17	47

Table S3. Simulation results obtained from Molecular Dynamics for the different NADES used in this work.

	BXylW	BTrehGlyW	BSorbW	BSucProW	BGly	PBS
ΔG_s [kcal/mol]	-32.92 ± 3.95	-28.12 ± 4.69	-24.89 ± 2.44	-24.84 ± 4.33	-28.01 ± 4.71	-31.28 ± 1.4
SASA [nm²]	173.45 ± 2.02	166.47 ± 4.02	160.79 ± 5.74	153 ± 2.87	156.95 ± 3.71	159.03 ± 2.72
R_g [nm]	2.1 ± 0.01	2.04 ± 0.02	2.02 ± 0.03	1.98 ± 0.02	2.00 ± 0.02	2.01 ± 0.03
RMSD [nm]	0.38 ± 0.07	0.31 ± 0.08	0.21 ± 0.15	0.18 ± 0.04	0.17 ± 0.04	0.21 ± 0.02
$D_{ARG38\text{-}HIS42}$ [nm]	0.66 ± 0.11	0.58 ± 0.03	0.58 ± 0.02	0.60 ± 0.12	0.65 ± 0.16	0.63 ± 0.12
$x_{hydrophobic}/x_{hydrophilic}$	1.077 ± 0.02	1.091 ± 0.01	1.106 ± 0.02	1.078 ± 0.03	1.083 ± 0.04	1.065 ± 0.02
$\Delta G_{s,hydrophobic}$ [kcal/mol]	31.93 ± 2.29	33.54 ± 2.48	35.96 ± 2.3	33.97 ± 2.15	35.08 ± 0.29	31.85 ± 1.8
H-Bonds [-]	653.41 ± 6.34	637.45 ± 17.12	592.67 ± 10.61	575.83 ± 18.11	574.85 ± 20.83	627.84 ± 9.01

Table S4. Simulation results obtained from Molecular Dynamics for the different BGly dilutions used in this work.

	BGly	BGly95	BGly90	BGly85	BGly80
ΔG_s [kcal/mol]	-28.01 ± 4.71	-31.39 ± 5.04	-25.06 ± 3.21	-28.9 ± 2.54	-25.82 ± 2.4
SASA [nm²]	156.95 ± 3.71	161.51 ± 3.7	155.98 ± 3.78	162.96 ± 4.36	161.35 ± 6.76
R_g [nm]	2 ± 0.02	2.02 ± 0.02	1.99 ± 0.02	2.02 ± 0.02	2.04 ± 0.05
RMSD [nm]	0.17 ± 0.04	0.41 ± 0.04	0.35 ± 0.07	0.27 ± 0.12	0.22 ± 0.15
$D_{ARG38\text{-}HIS42}$ [nm]	0.65 ± 0.16	0.64 ± 0.15	0.59 ± 0.07	0.58 ± 0.02	0.54 ± 0.02
$x_{hydrophobic}/x_{hydrophilic}$	1.083 ± 0.04	1.067 ± 0.03	1.117 ± 0.03	1.084 ± 0	1.105 ± 0.02
$\Delta G_{s,hydrophobic}$ [kcal/mol]	35.08 ± 0.29	29.12 ± 1.2	36.51 ± 2.47	34.01 ± 2.24	33.3 ± 1.45
H-Bonds [-]	574.85 ± 20.83	603.1 ± 20.37	581.98 ± 3.77	595.36 ± 18.27	606.84 ± 5.07

Table S5. Kirkwood Buff Integral between protein-cosolvent (G_{pc}) for the different NADES used in this work.

KBI_{pi} \ i =	***W***	***B***	***Gly***	***Xyl***	***Treh***	***Sorb***	***Suc***	***Pro***
Water	-26.02 ± 0.06		-	-	-	-	-	-
BXylW	-23.82 ± 0.07	-49.6 ± 0.7	-	-22.7 ± 0.7	-	-	-	-
BTrehGlyW	-24.05 ± 0.04	-48.9 ± 0.5	-28.2 ± 0.4	-	-34.7 ± 0.62	-	-	-
BSorbW	-24.12 ± 0.05	-47.9 ± 0.6	-	-	-	-30.0 ± 0.3	-	-
BSucProW	-26.32 ± 0.07	-48.2 ± 0.5	-	-	-	-	-26.7 ± 0.7	42.5 ± 0.7
100 wt%	-24.06 ±	-47.8 ±	-27.5 ±	-	-	-	-	-

BGly	0.04	0.2	0.7					
95 wt% BGly	-24.10 ± 0.14	-48.3 ± 1.0	-28.0 ± 0.3	-	-	-	-	-
90 wt% BGly	-24.14 ± 0.01	-47.5 ± 0.7	-28.1 ± 0.4	-	-	-	-	-
85 wt% BGly	-24.34 ± 0.16	-49.0 ± 0.8	-27.2 ± 0.7	-	-	-	-	-
80 wt% BGly	-24.41 ± 0.09	-48.8 ± 0.9	-28.1 ± 0.3	-	-	-	-	-

*W: water, B: betaine, Gly: glycerol, Xyl: D-(+)-xylose, Treh: trehalose, Sorb: D-sorbitol, Suc: sucrose, Pro: DL proline.

Table S6. Preferential interaction parameters between protein-cosolvent (Γ_{pc}) for the different NADES used in this work.

Gamma$_{pi}$ \ i =	***B***	***Gly***	***Xyl***	***Treh***	***Sorb***	***Suc***	***Pro***
BXylW	-23.4 ± 0.4	-	0.5 ± 0.3	-	-	-	-
BTrehGlyW	-10.4 ± 0.3	-2.6 ± 0.4	-	-2.5 ± 0.3	-	-	-
BSorbW	-14.3 ± 0.4	-	-	-	-3.5 ± 0.2	-	-
BSucProW	-12.4 ± 0.4	-	-	-	-	-0.1 ± 0.2	14.1 ± 0.2
100 wt% BGly	-17.4 ± 0.4	-4.7 ± 1.0	-	-	-	-	-
95 wt% BGly	-17.1 ± 1.0	-5.2 ± 0.5	-	-	-	-	-
90 wt% BGly	-15.4 ± 0.5	-5.0 ± 0.5	-	-	-	-	-
85 wt% BGly	-15.4 ± 0.6	-3.5 ± 1.0	-	-	-	-	-
80 wt% BGly	-14.2 ± 0.4	-4.1 ± 0.3	-	-	-	-	-

*W: water, B: betaine, Gly: glycerol, Xyl: D-(+)-xylose, Treh: trehalose, Sorb: D-sorbitol, Suc: sucrose, Pro: DL-proline.

40 **Supplementary Figures**

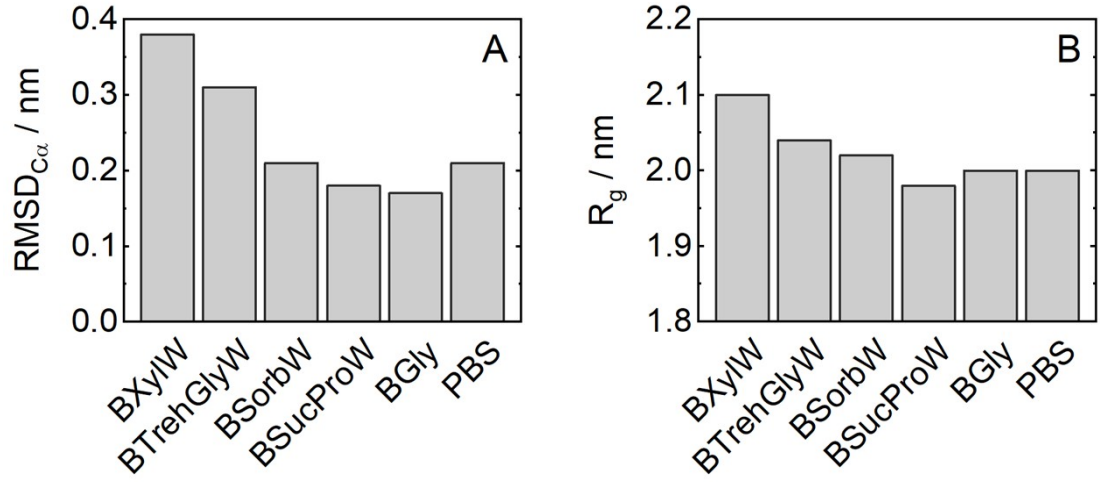

Figure S1 – (A) The root mean square deviation (RMSD) and (B) the radius of gyration (^{R_g}) for BetGly dilutions used in this work.

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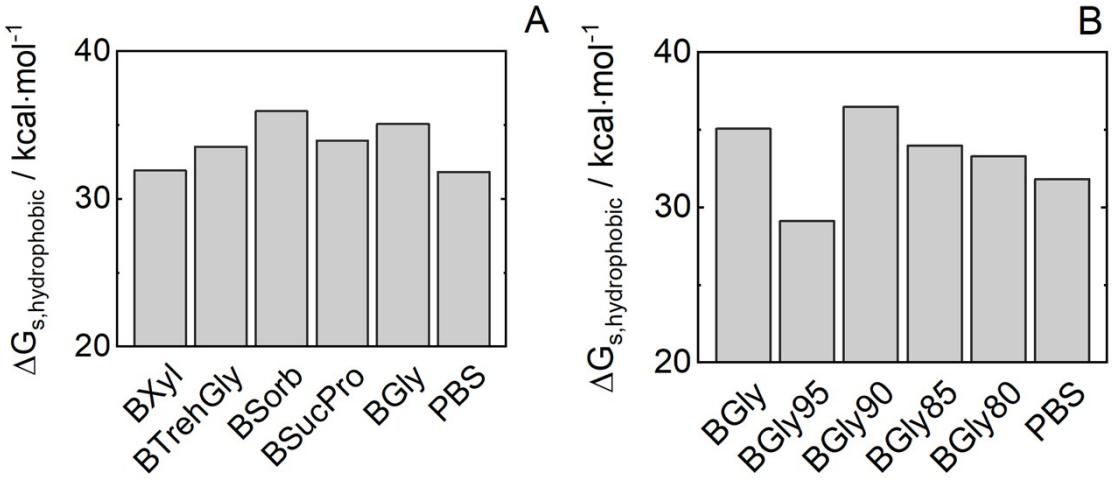

Figure S2 – The estimated solvation free energy ($^{\Delta G_s}$) of the hydrophobics groups for (A) NADES used in this work and (B) BGly dilutions.

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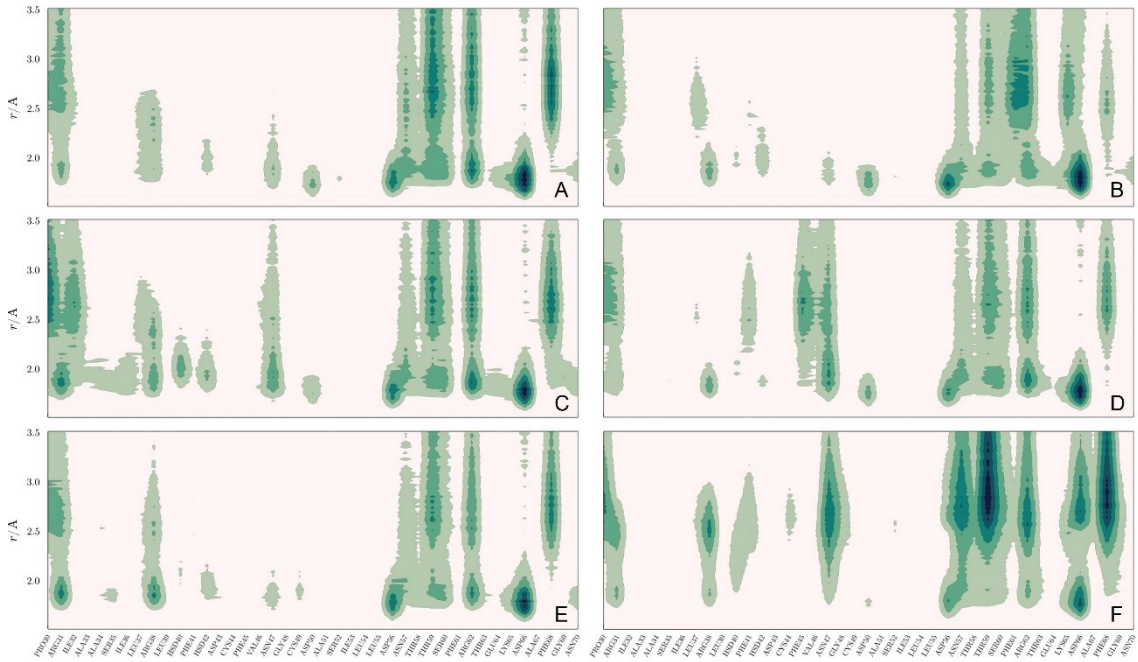

Figure S3 – Water minimum distance function density at the vicinity of active site residues. (A) water, (B) BTrehGlyW, (C) BSorbW, (D) BGly, (E) BXylW and (F) BSucProW

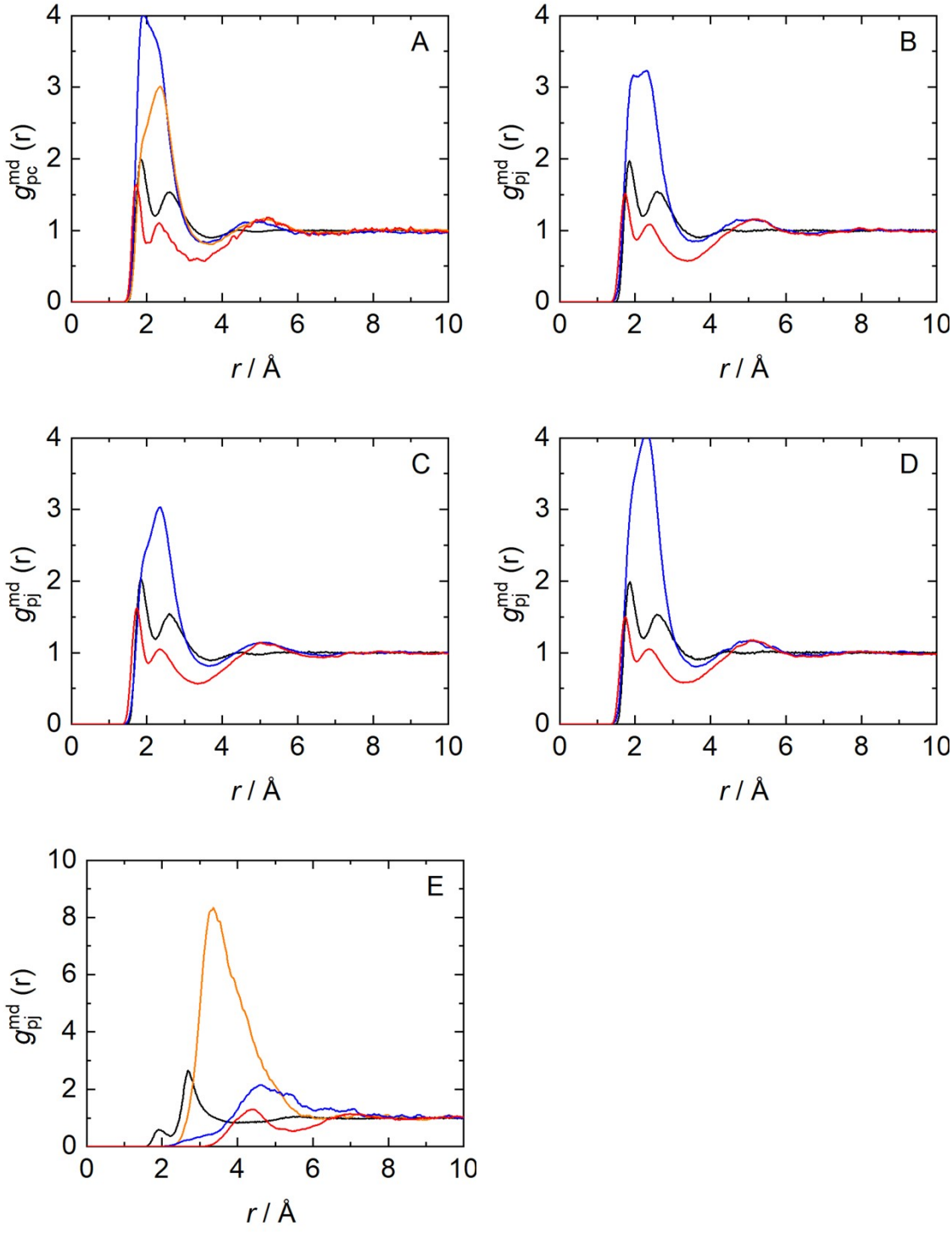

Figure S4 – Minimum-Distance Distribution Functions ($g^{md}_{pj}(r)$) between protein and solvent for: (A) BetTrehGlyW, (B) BSorbW, (C) BGly, (D) BXylW and (E) BSucProW. Solvents: water (black), betaine (red), second cosolvent (blue), third cosolvent (orange).

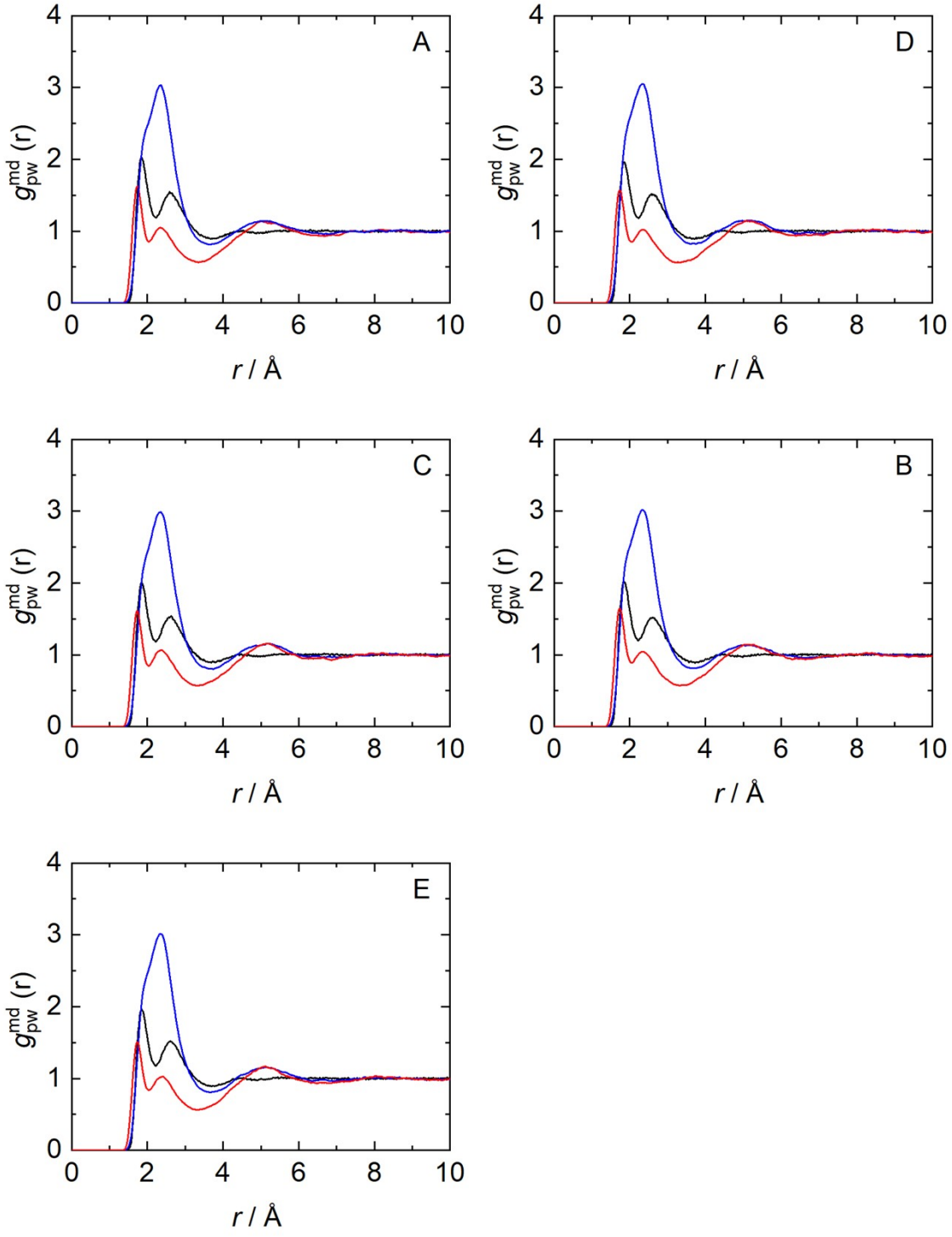

Figure S5 – Minimum-Distance Distribution Functions ($g_{pj}^{md}(r)$) between protein and solvent for: (A) BetGly, (B) BGly95, (C) BGly90, (D) BGly85 and (E) BGly80. Solvents: water (black), betaine (red), glycerol (blue).

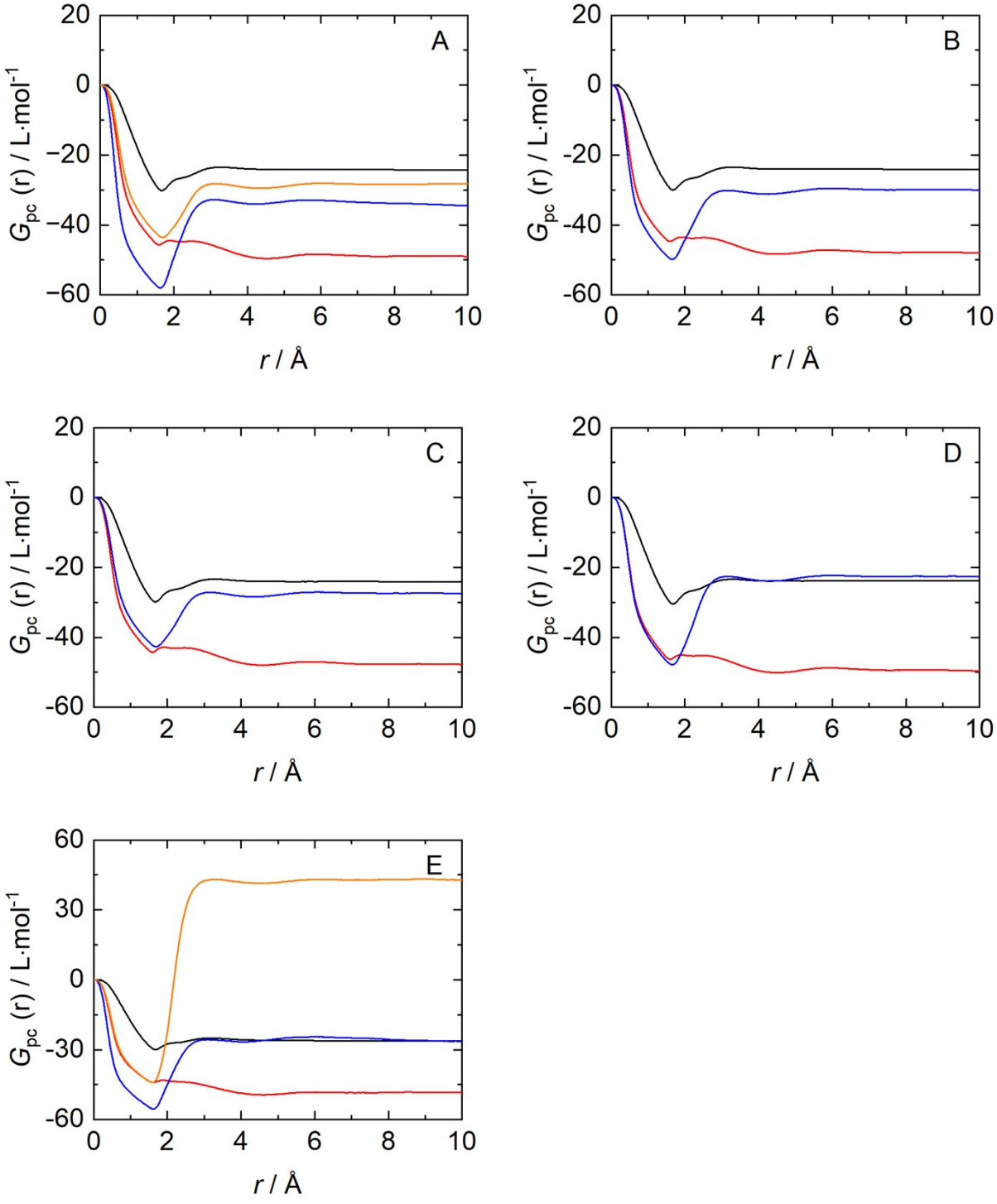

Figure S6 – Kirkwood Buff Integral profiles ($^{G_{pc}(r)}$) between protein and solvent for: (A) BetTrehGlyW, (B) BSorbW, (C) BGly, (D) BXylW and (E) BSucProW. Solvents: water (black), betaine (red), second cosolvent (blue), third cosolvent (orange).

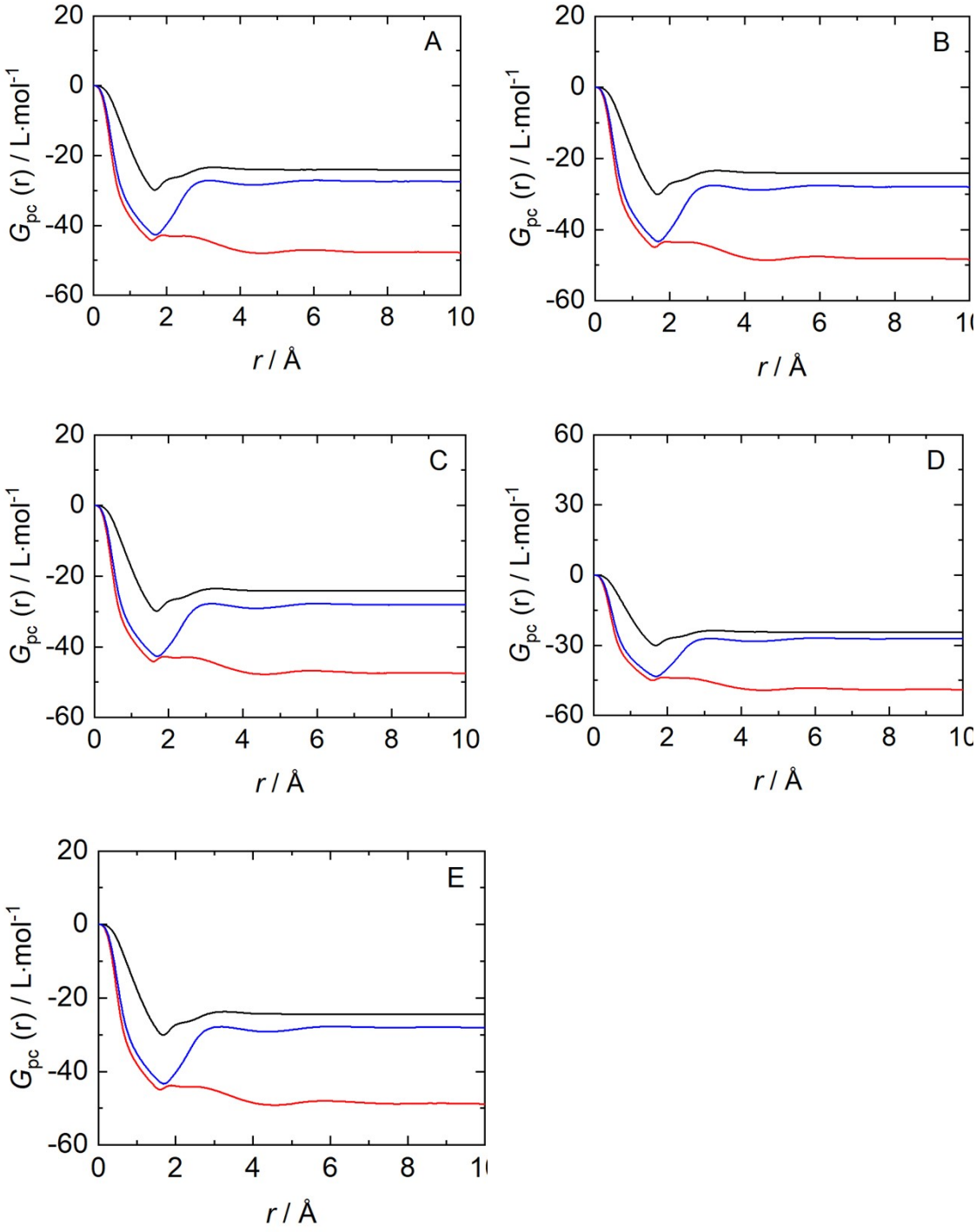

Figure S7 – Kirkwood Buff Integral profiles ($^{G_{pc}(r)}$) between protein and solvent for: (A) BetGly, (B) BGly95, (C) BGly90, (D) BGly85 and (E) BGly80. Solvents: water (black), betaine (red), glycerol (blue).

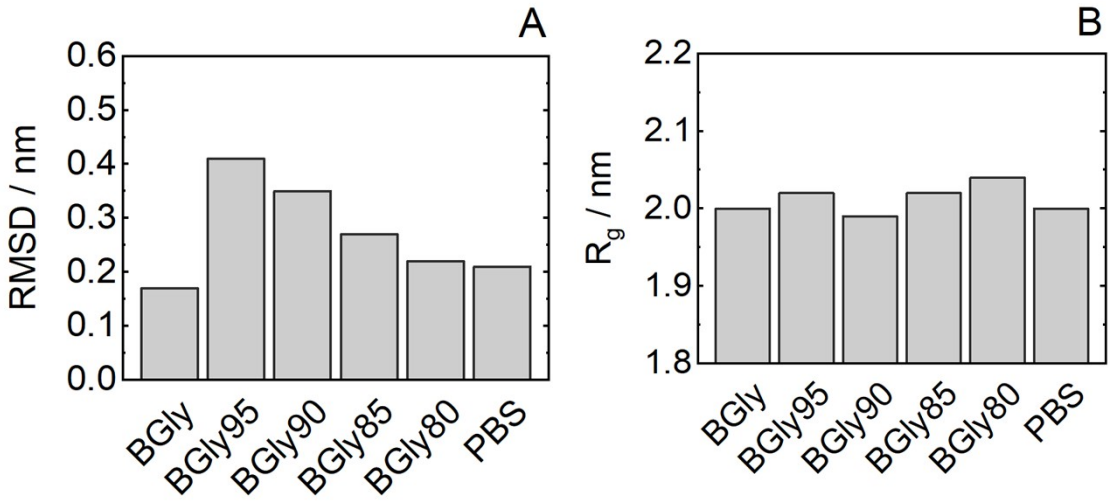

Figure S8 – (A) The root mean square deviation (RMSD) and (B) the radius of gyration (^{R_g}) for BetGly dilutions used in this work.

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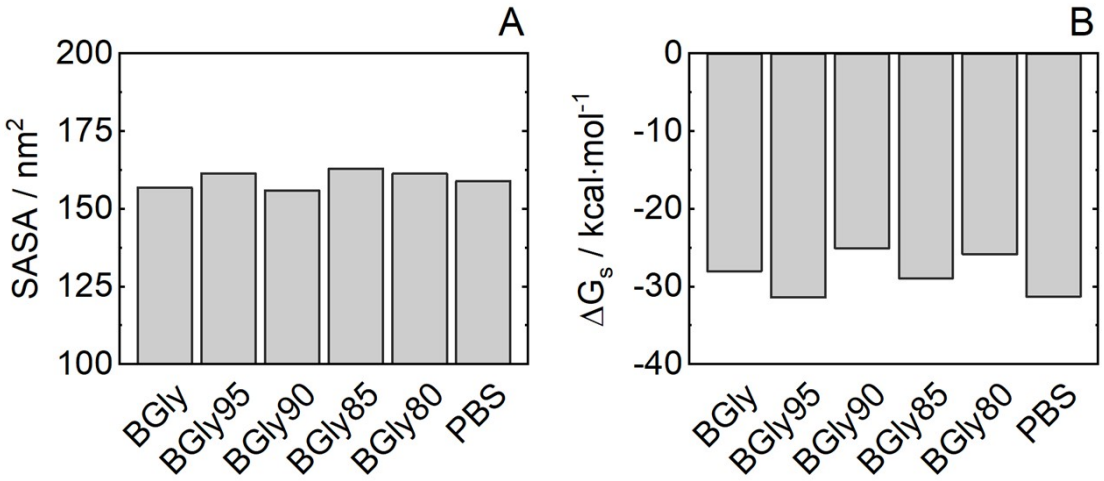

Figure S9 – (A) The solvent accessible surface areas (SASA) and (B) the estimated solvation free energy ($^{\Delta G_s}$) for BetGly dilutions used in this work.

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