Supplementary Materials

Stabilization of apo α -lactalbumin by binding of epi-gallocatechin-3-gallate: experimental and molecular dynamics study

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Figure S1. ApoALA and holoALA fluorescence quenching by EGCG. Emission spectra (excitation at 280 nm) of apoALA and holoALA (1 uM) in the presence of different concentrations of EGCG (1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 uM) at three pH values and two temperatures. pH: in phosphate buffer, pH 7.2, in 100 mM glycine-HCl, pH 2.5 and in 100 mM HCl (containing 34 mM NaCl), pH 1.2. Temperatures: at 6°C and 37°C.

A)

B)

Figure S2. Far UV (A) and near UV (B) CD spectra of holoALA and apoALA in 50 mM phosphate buffer pH 7.2.

A)

B)

Figure S3. Far UV (A) and near UV (B) CD spectra of holoALA and apoALA in 100 mM glycine buffer pH 2.5.

A)

B)

Figure S4. Far UV (A) and near UV (B) CD spectra of holoALA and apoALA 100 mM HCl (containing 34 mM NaCl), pH 1.2.

Table S1. The secondary structure content of apoALA without and with addition of EGCG (1:2) at three different pH.

Secondary structure	pH 1.2		pH 2.5		pH 7.2	
	apoALA	apoALA-EGCG	apoALA	apoALA-EGCG	apoALA	apoALA-EGCG
α -helix	39.60 ± 3.03^a	$37.97 \pm 1.01^{a,b}$	$36.17 \pm 0.91^{a,b}$	31.67 ± 2.65^b	$35.80 \pm 1.84^{a,b}$	$33.60 \pm 3.47^{a,b}$
β -sheet	6.27 ± 0.47^a	7.03 ± 1.07^a	8.07 ± 1.30^a	9.50 ± 2.17^a	7.07 ± 0.51^a	10.47 ± 4.58^a
β -turn	30.53 ± 1.87^a	30.53 ± 0.32^a	$29.13 \pm 1.27^{a,c}$	$28.33 \pm 0.76^{a,b}$	$27.07 \pm 1.11^{b,c}$	25.77 ± 1.18^b
random	23.60 ± 1.01^a	$24.43 \pm 0.31^{a,b}$	26.63 ± 0.40^b	30.53 ± 1.42^c	30.03 ± 1.16^c	30.20 ± 0.40^c

A)

B)

Figure S5. The first derivative of melting curves of 2.5 μM apoALA (A) and holoALA (B) in the absence and the presence of 50 μM EGCG in 50 mM phosphate buffer pH 7.2. Melting curves are obtained as temperature dependence of apoALA and holoALA fluorescence maxima (excitation at 280 nm).

Figure S6. Calculated RMSF (A), Rg (B) and RMSD (C) values from molecular dynamics simulation of holoALA and apoALA. In RMSF profiles amino acid residues involved in Ca^{2+} binding and regions with secondary structures are marked. Plots of Rg vs RMSD as a representation of the conformation space of holoALA and apoALA (D). Along 40 ns MDS, apoALA conformations have similar radius of gyration (Rg) and slightly lower root mean square deviation (RMSD) than holoALA, suggesting similar compactness.

Figure S7. Calculated RMSF (A), Rg (B) and RMSD (C) values from molecular dynamics simulation of holoALA and holoALA-EGCG. In RMSF profiles amino acid residues involved in Ca^{2+} binding, EGCG-interacting residues and regions with secondary structures are marked. Plots of Rg vs RMSD as a representation of the conformation space of holoALA and holoALA-EGCG (D). Concentrated population of holoALA-EGCG conformations, in contrast to more diffuse holoALA population, implies that conformational changes of holoALA-EGCG during MDS were much more subtle than of holoALA.

Figure S8. Calculated RMSF (A), Rg (B) and RMSD (C) values from molecular dynamics simulation of apoALA and apoALA-EGCG. In RMSF profiles amino acid residues involved in Ca^{2+} binding, EGCG-interacting residues and regions with secondary structures are marked. Plots of Rg vs RMSD as a representation of the conformation space of apoALA and apoALA-EGCG (D). Slightly less diffuse population of conformations of apoALA-EGCG, in comparison to apoALA, indicate narrower range of conformational changes in the presence of EGCG, additionally supporting EGCG-stabilizing effect.

Fig S9. Time-profiles of RMSD for specific residues obtained by 40 ns molecular dynamics simulation of holoALA (A), holoALA-EGCG (B), apoALA (C) and apoALA-EGCG (D). In apoALA instability of Ca²⁺-binding region is reflected in increase of RMSD of this region (less navy and more green and redish area), and EGCG binding to apoALA stabilize this region resulting in reduction of RMSD (less green and absence of red area).

Fig S10. 2D RMSD plots obtained by 40 ns molecular dynamics simulation of holoALA (**A**), holoALA-EGCG (**B**), apoALA (**C**) and apoALA-EGCG (**D**). EGCG binding to apoALA stabilize it resulting in 2RMSD with more low RMSD area (green area with RMSD 0.75-1.25) and less high RMSD area (yellow area with RMSD 1.3-1.7 and red area with RMSD 1.7-2.1). When EGCG is bound, apoALA has more areas with lower RMSD (0.7-1.2 Å) and less areas with higher RMSD (1.5-2 Å), suggesting more similar conformations during simulation time and less vivid conformational changes of apoALA when EGCG is present.

Fig S11. Distance of salt bridge formed between Lys79 and Asp82 during 40 ns molecular dynamics simulation of holoALA, apoALA and apoALA-EGCG. Inside of Ca^{2+} -binding loop, relatively long-term salt bridge Lys79-Asp82 in holoALA (about 60% of MDS time) is minimized to about 5 % of time intervals in apoALA. EGCG binding restores longevity of this salt bridge (about 45% of time) therefore contributing to stability of Ca^{2+} -binding region.

Fig S12. The mutual distances between Ca^{2+} -ligating atoms Ca^{2+} -binding residues, Lys79, Asp82, Asp84, Asp87 and Asp88 during 40 ns molecular dynamics simulation of holoALA (A), apoALA (B) and apoALA-EGCG (C). In comparison to holoALA, the mutual distances between Ca^{2+} -ligating atoms in apoALA are increased, while the presence of EGCG mostly shorten back these distances sticking them together.

Fig S13. Average mutual distances between Ca^{2+} -binding residues, Lys79, Asp82, Asp84, Asp87 and Asp88 during 40 ns molecular dynamics simulation of holoALA, apoALA and apoALA-EGCG. **A)** Average mutual distances between Ca^{2+} -ligating atoms of Ca^{2+} -binding residues; **B)** Average mutual distances between Ca atoms of Ca^{2+} -binding residues.

A) **B)** **C)**

Fig S14. Distance between Tyr103 of α -lobe and Gln54 and Asn56 of β -lobe during 40 ns molecular dynamics simulation of holoALA (**A**), apoALA (**B**) and apoALA-EGCG (**C**). Tyr103-Gln54 and Tyr103-Asn56 distances are lower than 2.5 Å in holoALA during most of simulation time, in contrast to apoALA where these distances are < 2.5 Å only in few very short time periods, therefore not allowing direct hydrogen bonds. EGCG binding reverts back short Tyr103-Gln54 and Tyr103-Asn56 distances during most of the time.