Electronic Supporting Information

Gastric stability of bare & chitosan-fabricated ferritin and its bio-mineral: Implication towards potential dietary iron supplement

Rohit Kumar Raut, Gargee Bhattacharyya, Rabindra K. Behera*

Department of Chemistry, National Institute of Technology, Rourkela - 769008, Odisha, India.

*To whom correspondence should be addressed: Rabindra K. Behera, Tel: +91-661-2462980; Fax: +91-661-2462651; E-mail: beherarabi@nitrkl.ac.in

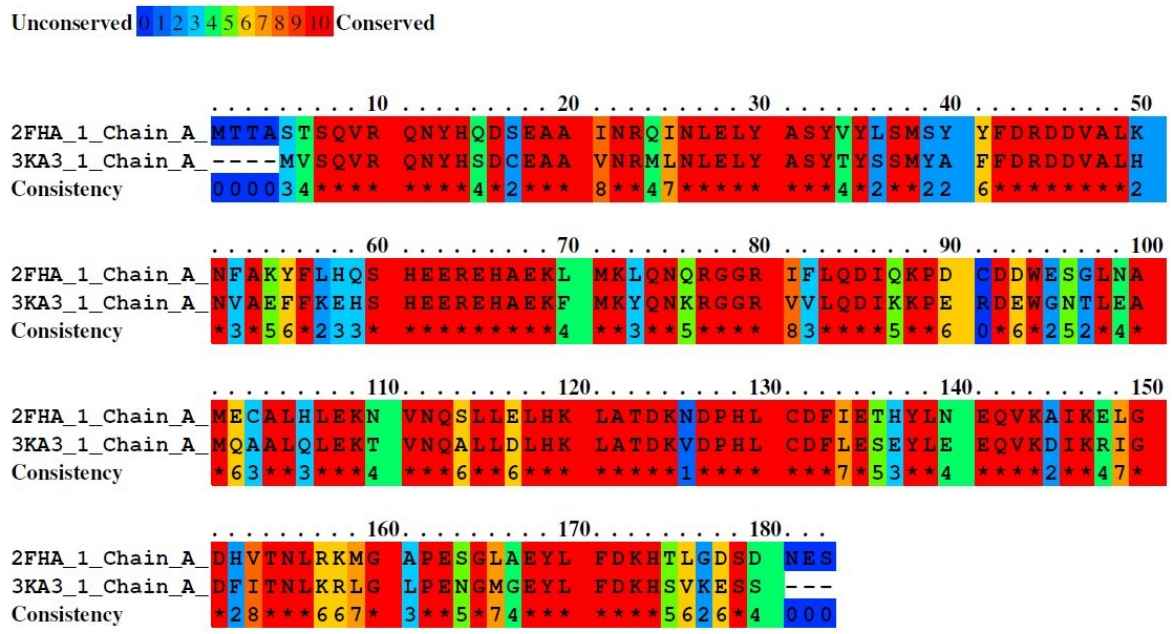

Figure S1: Sequence alignment of bullfrog M ferritin (1MFR) and human H chain ferritin (2FHA). The current colour scheme of the alignment is for amino acid conservation. The conservation scoring is performed by PRALINE. The scoring scheme works from 0 up to 10 for the least to the most conserved alignment position respectively. Amino acid position (numbering) is based on human H chain ferritin.

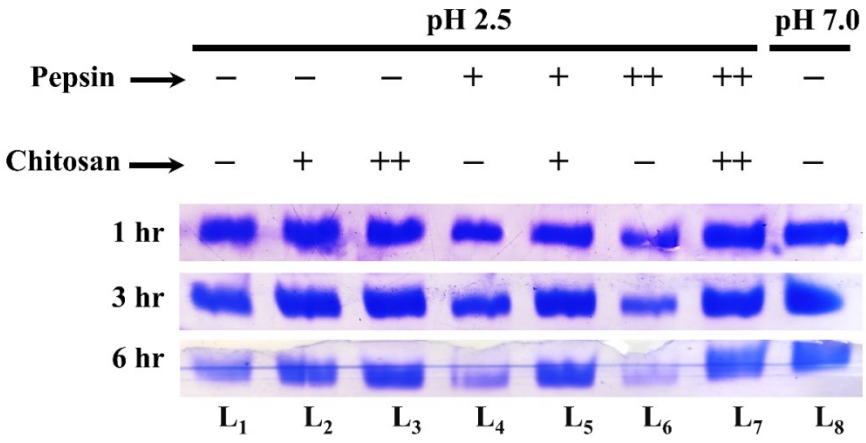

Figure S2: Effect of chitosan on stability of ferritin protein cage under gastric conditions. Native PAGE analysis for the digestive stability of bare apo-ferritin samples (1 mg/mL), where (+) and (++) indicate the concentration of chitosan and pepsin at 1 mg/mL and 2 mg/mL respectively. The (−) signs indicate the absence of chitosan/pepsin. L_1 and L_8 are the control reactions (in the absence of pepsin).

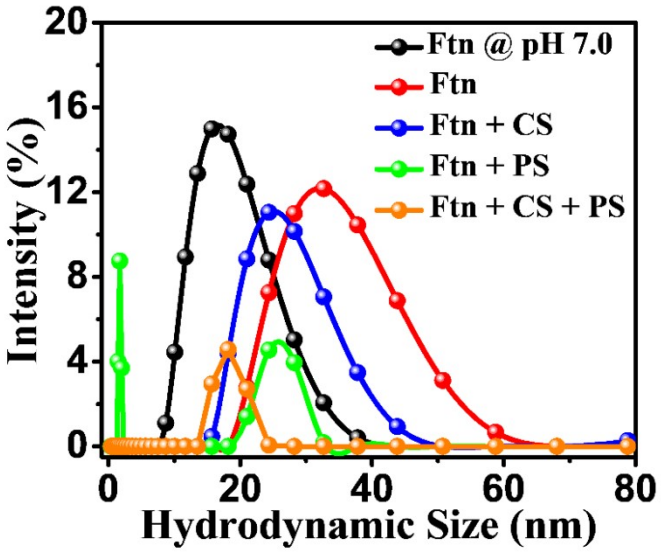

Figure S3: Effect of pepsin on cage integrity of bare and chitosan-fabricated ferritin samples under gastric condition. DLS (Intensity distribution) based hydrodynamic sizes (diameter) of bare and chitosan-fabricated ferritin complex. Ferritin, chitosan and pepsin were maintained at same concentration i.e. 0.25 mg/mL. All the samples were incubated at pH 2.5, except in control (in black).

Table S1: The DLS based size analysis of bare and chitosan-coated apo ferritin samples. All the samples were incubated at pH 2.5, except in control (ferritin at pH 7.0).

Samples	Number Distribution (nm)	Intensity Distribution (nm)
Ferritin @ pH 7.0	10.1 ± 0.1	14.9 ± 1.1
Ferritin @ pH 2.5	11.7 ± 0.2	30.7 ± 2.8
Ferritin + Chitosan @ pH 2.5	21.2 ± 0.3	22.9 ± 2.1
Ferritin + Pepsin @ pH 2.5	1.9 ± 0.2	1.9 ± 0.3, 24.2 ± 0.2
Ferritin + Chitosan + Pepsin @ pH 2.5	14.6 ± 1.5	15.9 ± 0.1

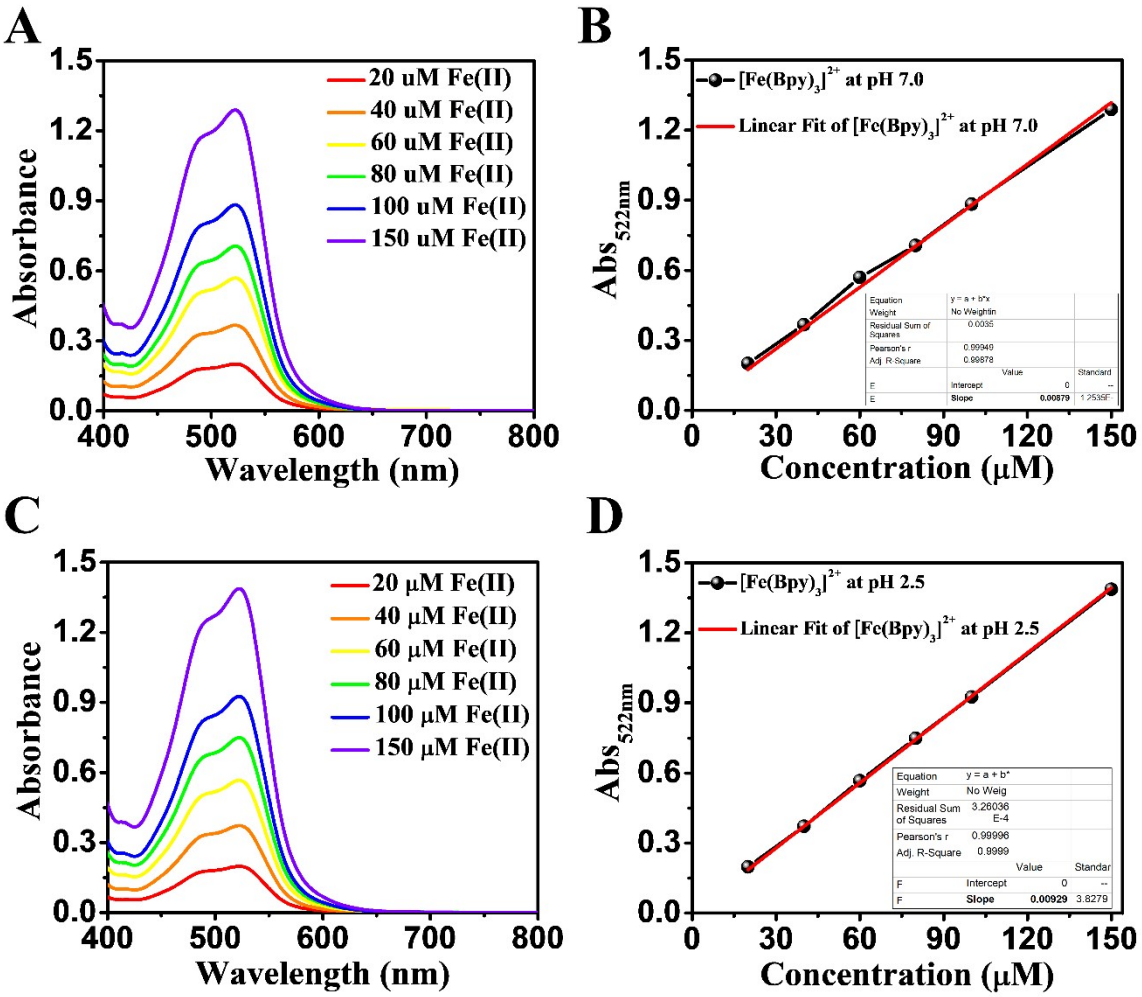

Figure S4: Determination of molar absorptivity for the Fe(II)-Bpy complex at pH 7.0 and pH 2.5. The absorption spectra were obtained by monitoring the formation of Fe^{2+}-Bpy complex at 522 nm at pH 7.0 and pH 2.5.

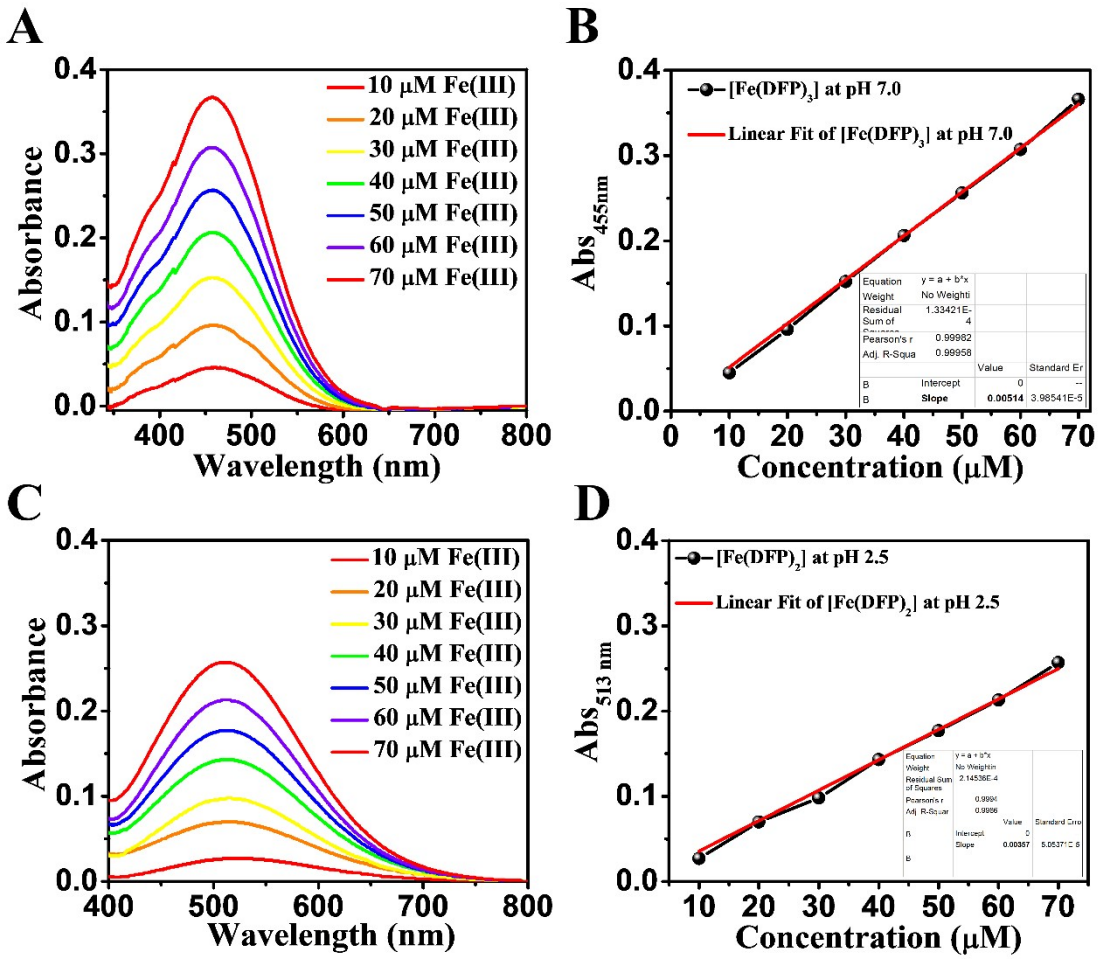

Figure S5: Determination of molar absorptivity for the Fe(III)-DFP complex at pH 7.0 and pH 2.5. The absorption spectra were obtained by monitoring the formation of Fe^{3+}-DFP complex at 455 nm and 513 nm at pH 7.0 and pH 2.5 respectively.

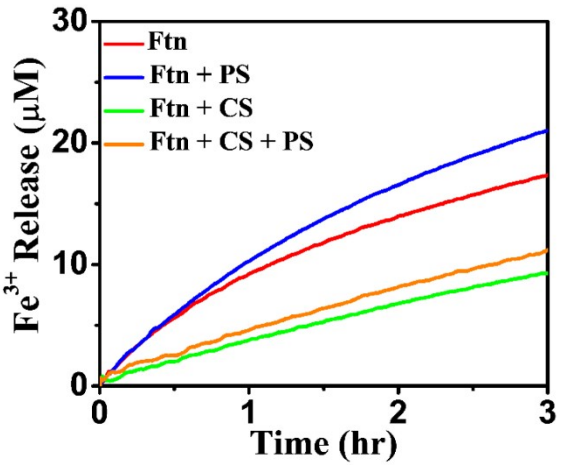

Figure S6: Effect of chitosan on non–reductive iron mobilization under gastric condition. Non-reductive iron mobilization facilitated by deferoxamine (DFO, Fe^{3+} chelator) from bare and chitosan-coated mineralized ferritin under gastric conditions. The kinetics of Fe^{3+} mobilization was obtained by monitoring formation of Fe^{3+}-DFO complex at 425 nm at pH 2.5.

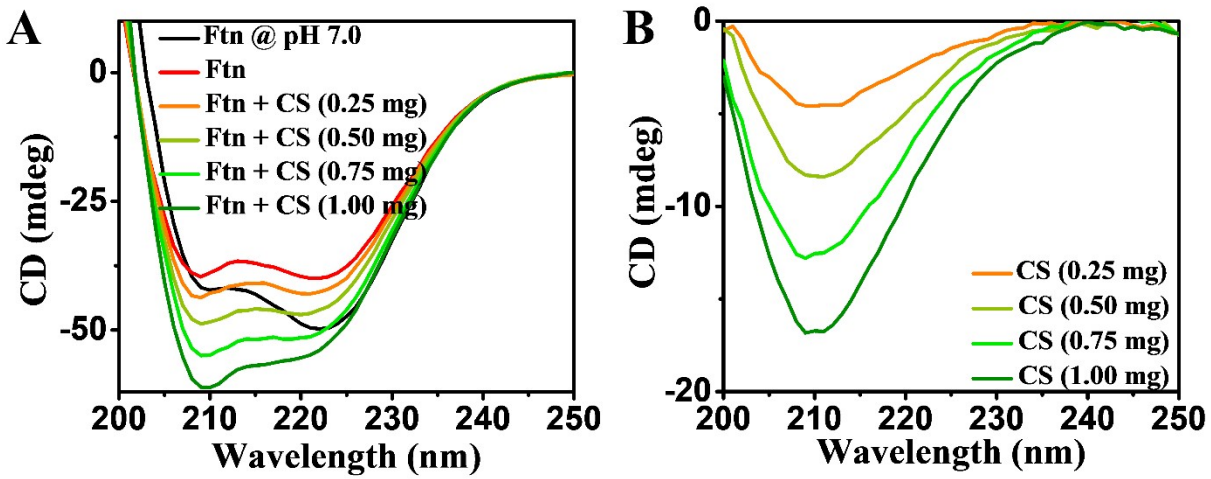

Figure S7: Effect of chitosan and pepsin on ferritin protein structure and stability. Circular Dichroism spectra of bare and chitosan-coated ferritin samples; **(A)** Interaction study between ferritin and chitosan (0.25 – 1 mg/mL). **(B)** CD spectra of chitosan (0.25 – 1 mg/mL) Both experiments were carried out in pH 2.5 in the absence of pepsin.

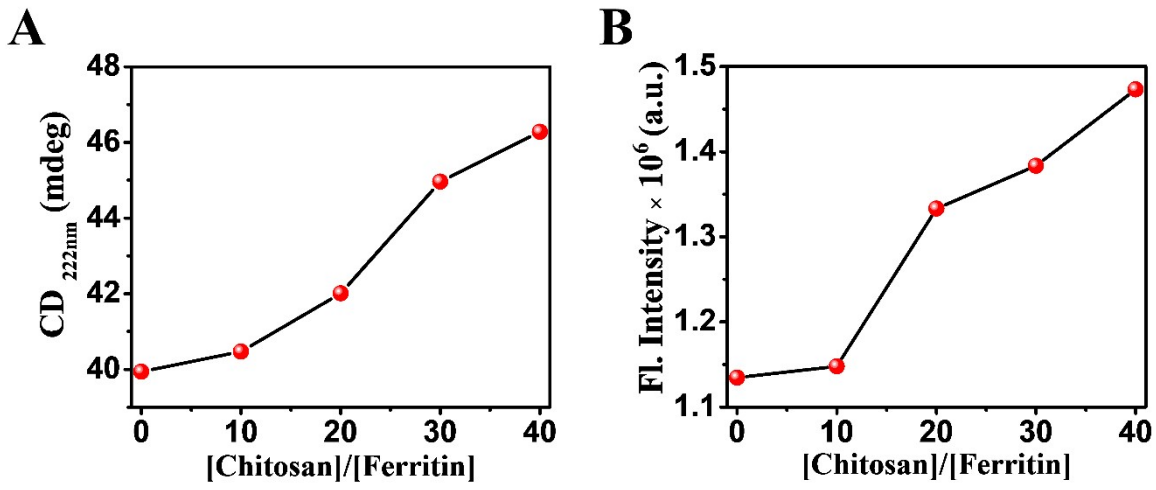

Figure S8: Restoration of CD and Fluorescence signal of ferritin upon chitosan titration. **A.** CD signal at 222 nm and **B**. fluorescence intensity at 330 nm plotted as a function of [Chitosan]/[ferritin] molar ratios at gastric pH 2.5.

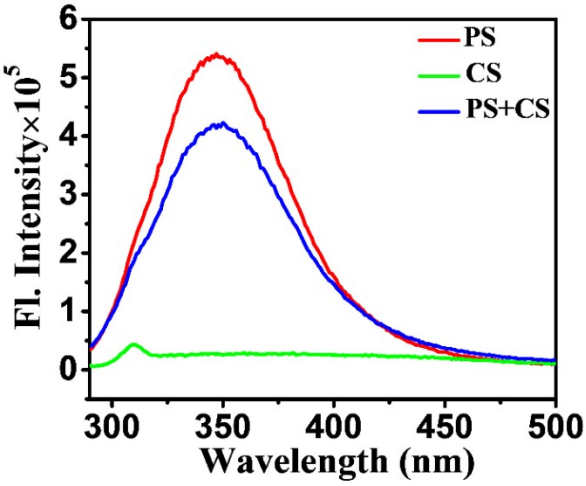

Figure S9: Fluorescence analysis of chitosan-pepsin interaction. Emission spectra (λ_{ex} 280 nm) of pepsin and chitosan at 1:1 ratio in gastric pH 2.5.

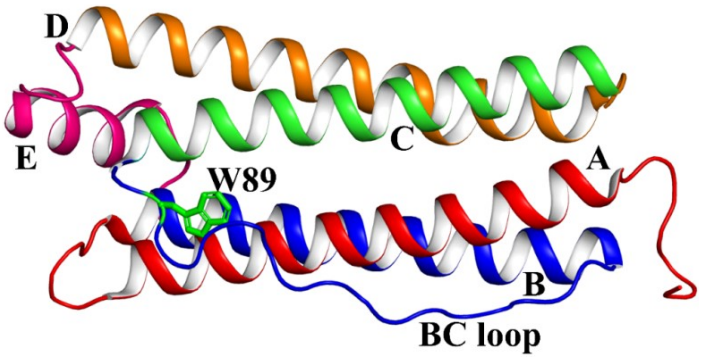

Figure S10: Cartoon representation of frog M ferritin subunit, where tryptophan (W) (green color) located at 89 position in the sequence (BC loop) is highlighted in stick form. The image was generated using PyMOL (**PDB ID**-3KA3).

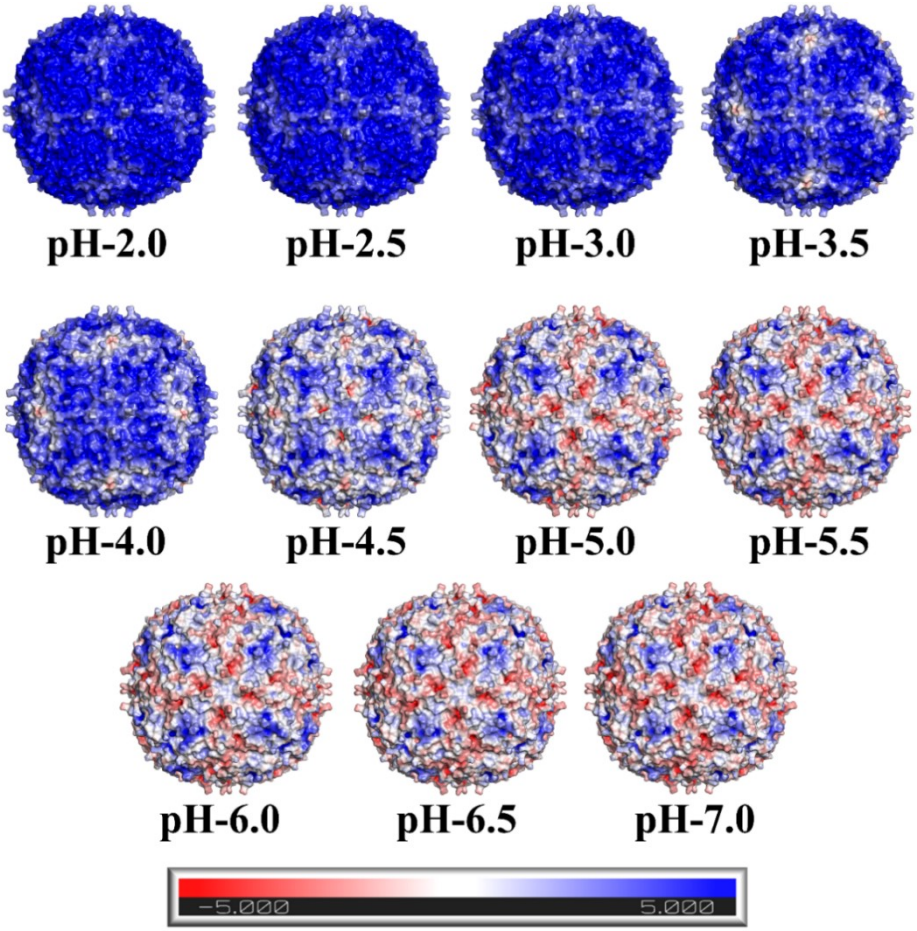

Figure S11: Effect of pH on the surface electrostatics of ferritin protein. pH dependent electrostatic analysis of frog M ferritin (**PDB:**1MFR). The surface electrostatics (the electrostatic potential expressed in the units of ± 5 k_BT/e) is generated by using ABPS tool in PyMOL. The blue, white and red shades represent the presence of positively, neutrally and negatively charged amino acid residues respectively.

RAW DATA FOR FIGURE - 2, 3, 6, S2

Figure-2

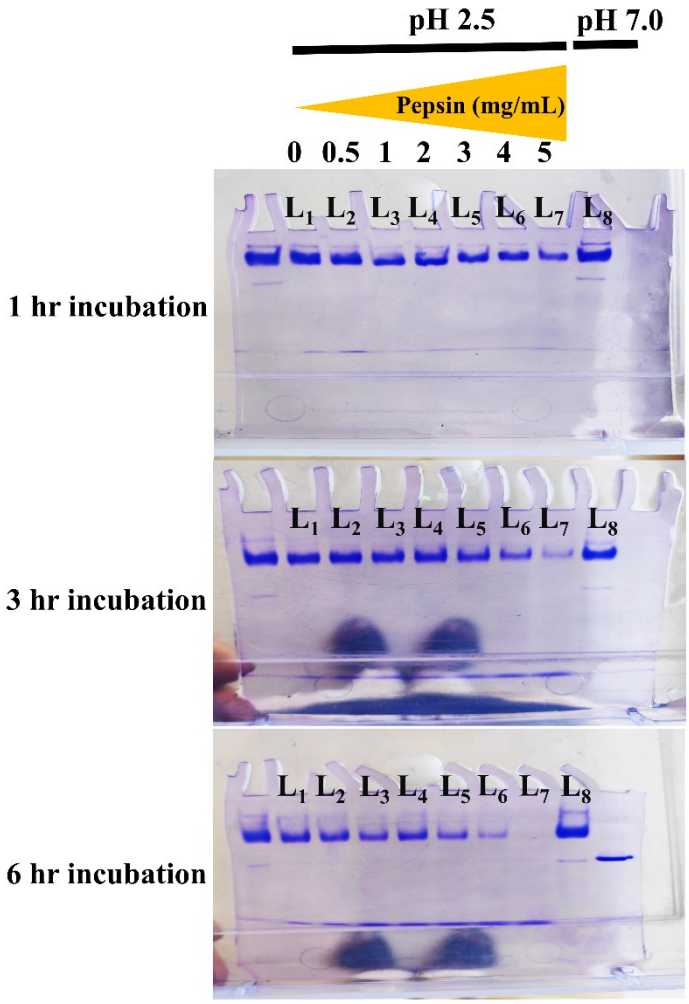

Figure-2: Effect of gastric pH and pepsin concentration on cage integrity of ferritin protein: Native PAGE analysis for the digestive stability of apo–ferritin against pH 2.5 and increasing concentration of pepsin (0 – 5 mg/mL; L_1 to L_7). Control (L_8): apo–ferritin sample at pH 7.0 in the absence of pepsin. Ferritin concentration was maintained at 1 mg/mL in the reaction solutions.

Figure-3: Prussian (Iron) Staining

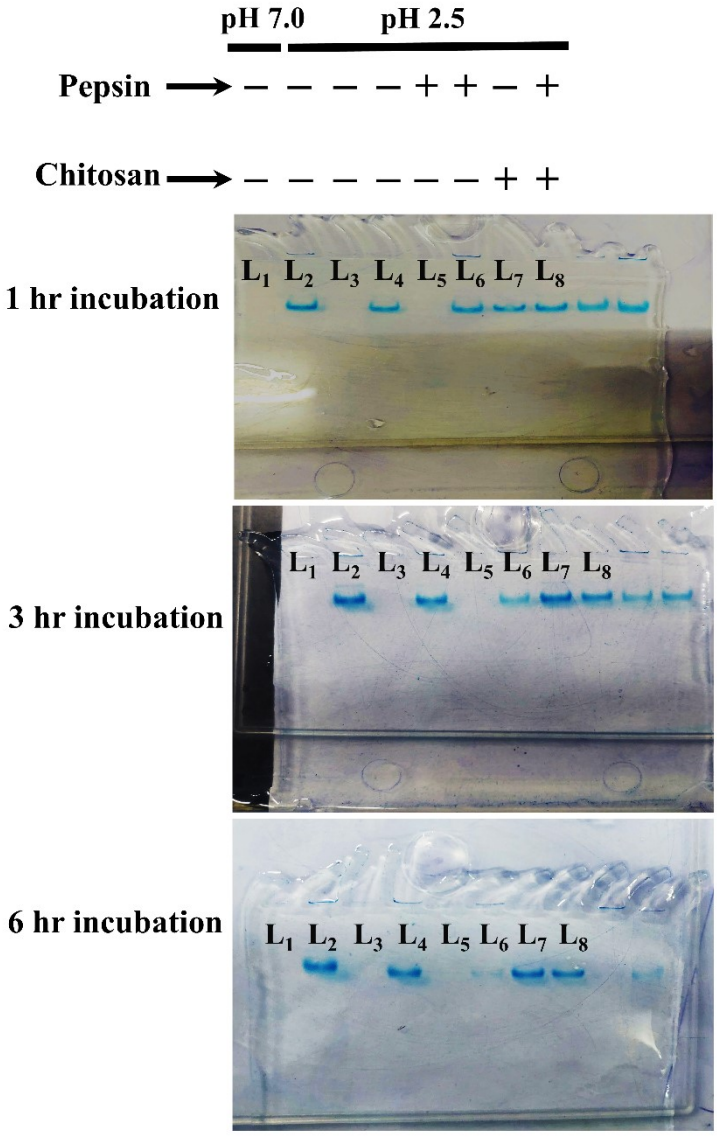

Figure-3: Coomassie (Protein) Staining Data

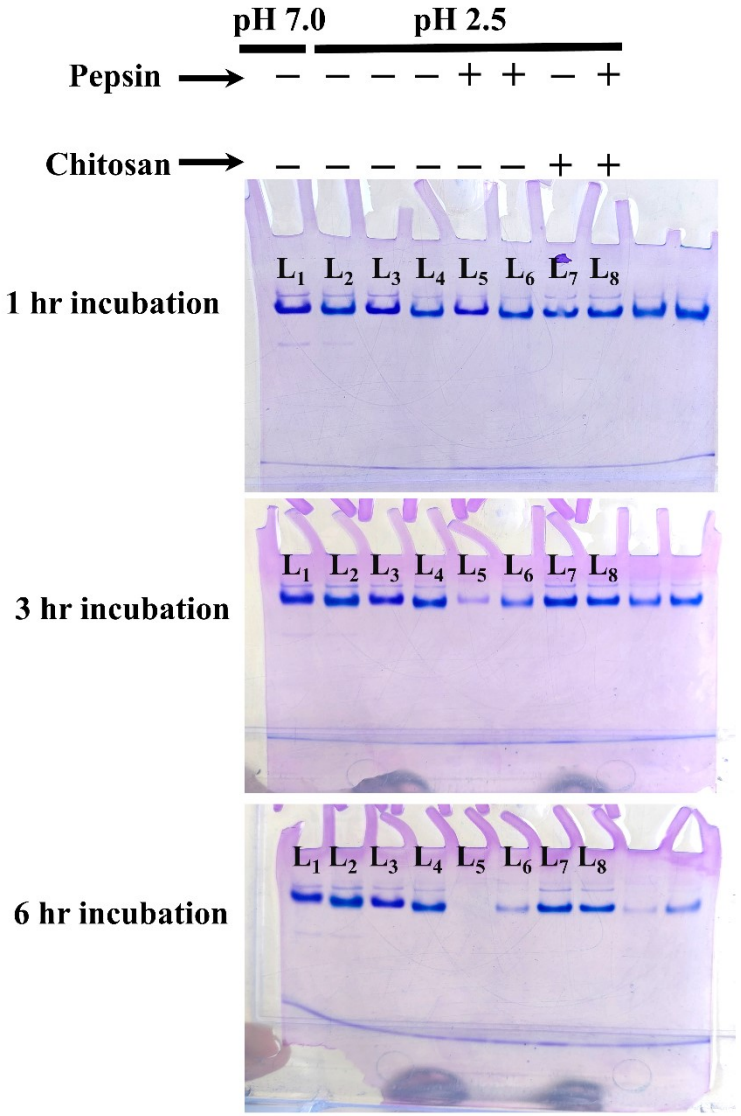

Figure 3: Native PAGE analysis of digestive stability of bare and chitosan-fabricated ferritin protein cage and its iron bio–mineral under gastric conditions. Apo and mineralized ferritin protein samples were run in a 5% (w/v) native gel at 100 V for 1.5 hr and was treated with acidified $K_4Fe(CN)_6$ solution to visualize the encapsulated ferric iron mineral (by formation of Prussian blue precipitate) followed by visualization of ferritin protein cage by Coomassie staining.

Figure-6

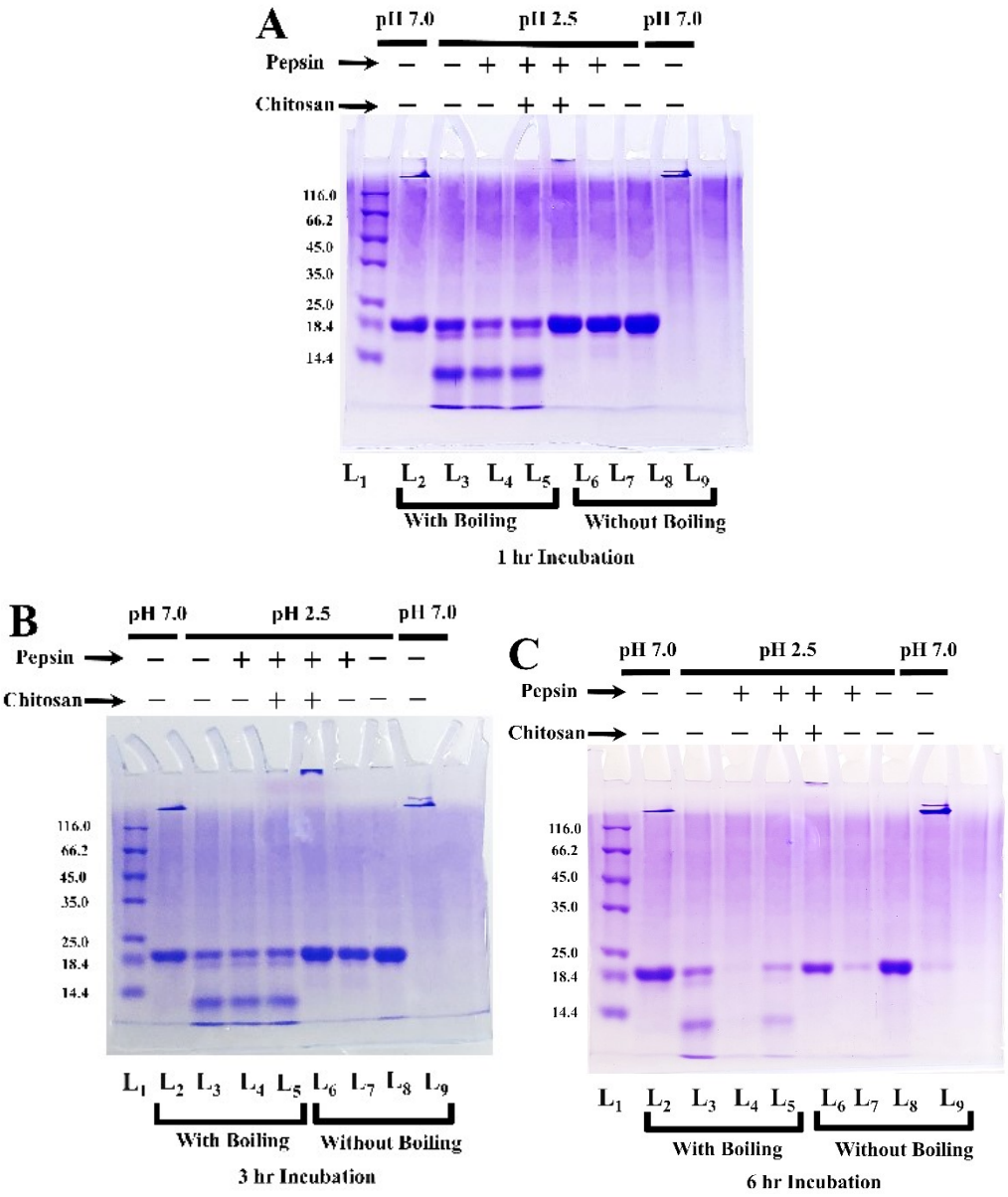

Figure 6: Analysis of SGF treated bare and chitosan-fabricated ferritin degradation by SDS-PAGE. The fragmentation pattern of bare and chitosan-modified ferritin complex in presence and absence of pepsin at pH 2.5 analyzed with boiling (L_2, L_3, L_4, L_5 : samples were boiled for 15 mins before loading) and without boiling (L_6, L_7, L_8, L_9) for three different incubation periods: **(A)** 1hr, **(B)** 3hr, and **(C)** 6hr. L_2 & L_9 – ferritin at pH 7.0 taken as control. Concentrations of ferritin, chitosan and pepsin were maintained at 1 mg/mL in the reaction mixture. (+) and (−) signs represent the presence and absence of chitosan/pepsin. L_1: protein ladder.

Figure-S2

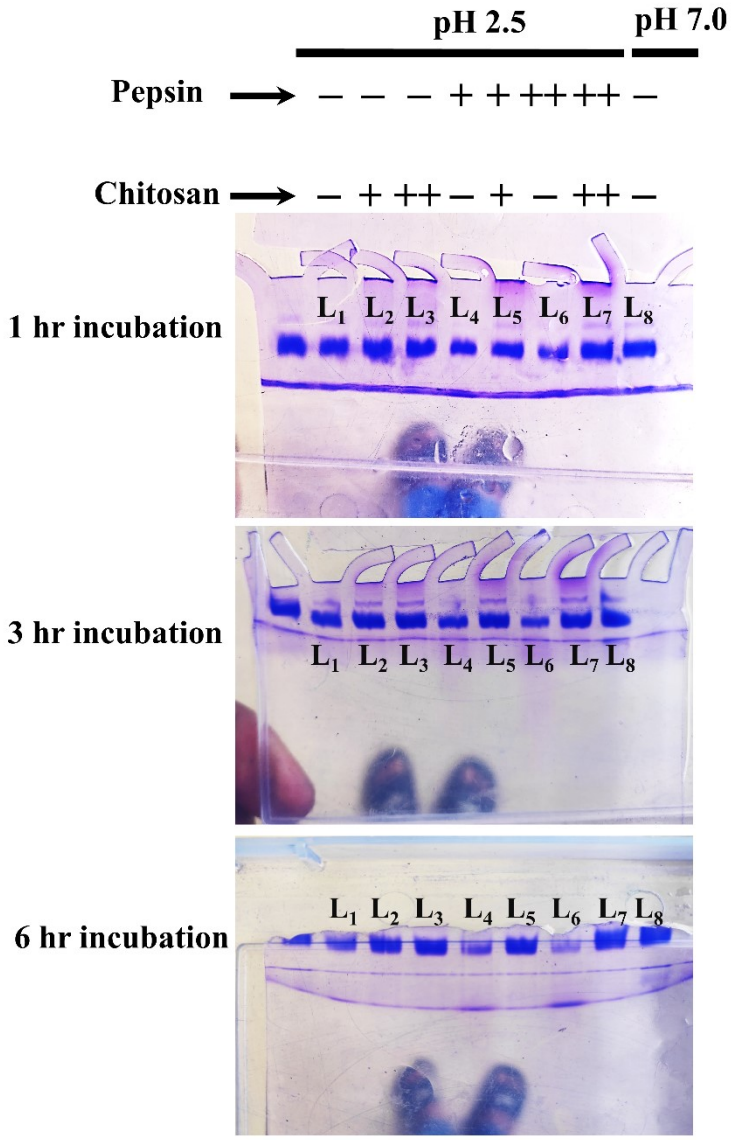

Figure S2: Effect of chitosan on stability of ferritin protein cage under gastric conditions. Native PAGE analysis for the digestive stability of bare apo-ferritin samples.