Nanoformulation approach for improved antimicrobial activity of bovine lactoperoxidase

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SUPPLEMENTARY FILE

Materials and Methods

1. Purification of LPO

LPO was purified from the fresh bovine mammary gland secretions, the samples were skimmed and separated from fat. These were diluted twice with 50 mM Tris-HCl (pH 7.8). Cation exchanger CM-Sephadex C-50 (7 g/liter) equilibrated in 50 mM Tris-HCl, pH 7.8, and stirred slowly for about 1 h with a mechanical stirrer. The gel was allowed to settle, and the solution was decanted. The protein-bound gel was washed with an excess of 50 mM Tris-HCl (pH 7.8) to remove the unbound proteins. The washed gel was loaded on a CM-Sephadex C-50 (Amersham Biosciences) column (10x2.5 cm) and equilibrated with 50 mM Tris-HCl (pH 7.8). The elution of LPO was done with 0.2 M NaCl using the same buffer. The protein was concentrated using an Amicon ultrafiltration cell. The concentrated protein sample was passed through a Sephadex G-200 column using 50 mM Tris-HCl buffer, pH 7.8. The elution was done at a flow rate of 6.0 ml/h, lyophilized, and stored at 253 K.

2. Preparation of LPO nanoparticles

LPO nanoparticles were prepared by dissolving 0.2% (w/v) of bLPO powder in phosphate buffer saline (PBS) under agitation for 1 hour at 25°C. After 1 hour of agitation, bLPO solution was adjusted to different pH values (4, 7 and 10) and kept at different thermal conditions (60-90°C) varying holding time (0- 60 min). The most favorable condition for

LPO nanoparticle formation was found at pH 7, holding time of 20 min at 75°C (Bengoechea *et al*., 2011).

3. Characterization of LPO Nanoparticles

The particle size distribution and surface morphology of LPO nanoparticles were measured using dynamic light scattering (DLS) and TEM respectively. For TEM analysis, LPO nanoparticles were resuspended in 0.01 M PBS (pH 7.4). The particles were then placed on carbon-coated copper grid, air-dried for 2 min. and negatively stained using 1% aqueous solution of uranyl acetate for 1 min. Further, samples were examined using transmission electron microscopy (TEM, Talos L120C, Thermo Fisher, Waltham, MA, USA) for morphology. DLS measures the intensity of the light scattered over time. It has also reconfirmed the stability and size of LPO nanoparticles having particle diameter relatively similar with TEM results.

4. Antibacterial Studies

4.1.Medium and Bacterial strains

The bacterial strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States). A differential medium xylose-lysine deoxycholate agar (XLD agar) was used for the cultivation of *Shigella flexneri.* Luria–Bertani (LB agar) medium was used for the cultivation of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Mueller Hinton broth (MHB) was used for antimicrobial susceptibility testing of proteins. Kanamycin was acquired from Sigma Aldrich (India). All the chemicals were analytic grade chemicals and Media components were acquired from Himedia (India).

4.2.Measurement of Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined using a standard serial dilution broth method for lactoperoxidase (LPO) and LPO nanoparticles against *S. flexneri, P. aeruginosa, S. aureus,* and *E. coli.* For this 24 hour culture of bacterial strain was prepared. After preparation of 0.5 McFarland bacterial suspension in Muller Hinton Broth (MHB) medium, highest to the lowest concentration of LPO and LPO nanoparticles was added in each well of a 96-well microtitre plate. The protein concentration in the wells ranged from 2mg to 0.019µg. After incubation at 37℃ for 24 h, the absorbance at 600 nm for LPO and its nanoparticles were measured.

4.3.Growth Inhibition Curve

The effect of LPO and LPO nanoparticles on the growth of *S. flexneri P. aeruginosa, S. aureus, E. coli* was examined. Bacteria were cultured at 37℃, agitation (200 rpm). The culture was grown in Muller Hinton broth (MH Broth) to an O.D600 of 0.1, and then equally allocated into 96 well microtitre plates (200µl/well). Then LPO and its Nanoparticles were added to their MIC concentration. Kanamycin was used as a positive control, and the culture without the protein was used as bacterial growth control. The absorbance was recorded at 600nm was recorded by a microplate spectrophotometer (Epoch, Biotek, USA) at 1hr interval.

4.4.Agar Disc Diffusion Assay

The method of Bauer et al (Kirby-Baure *et al*., 1966) was used to assess the antimicrobial activity of LPO and LPO nanoparticles. Bacterial cultures of *S. aureus, E. coli, Pseudomonas aeruginosa, and Shigella flexneri* were grown at their optimum temperature (37°C) overnight in a muller Hinton broth medium. Thereafter, the

bacteria were diluted to about 105 colony forming units (CFU)/mL. The protein minimum inhibitory concentration (MIC) was loaded onto sterile papers (10mm diameter) and placed on the MHA (Muller Hinton Agar) surface. Plates were incubated at the optimal temperature for each strain for 18-24 hrs. The diameter of the bacterial inhibition zone indicated the antibacterial activity.

Table S1. The minimum inhibitory concentration of free lactoperoxidase and LPO nanoparticles.

S.no	**Protein (mg)**	***S. aureus***	***P. aeruginosa***	***S. flexineri***	***E. coli***
1.	LPO	0.031	0.125	0.8	0.031
2.	LPO Nanoparticles	0.031	0.031	0.5	0.015

Table S2. Zone of inhibition of free LPO and LPO nanoparticles.

S.no	**Bacterial strains**	**LPO (ZOI in mm)**	**LPO nanoparticles (ZOI in mm)**
1.	*Staphylococcus aureus*	22	25
2.	*Shigella flexineri*	15	17
3.	*E. coli*	13	17
4.	*Pseudomonas aeruginosa*	15	17

Figure S1. Diagram illustrating the biological activities of the LPO nanoparticles.

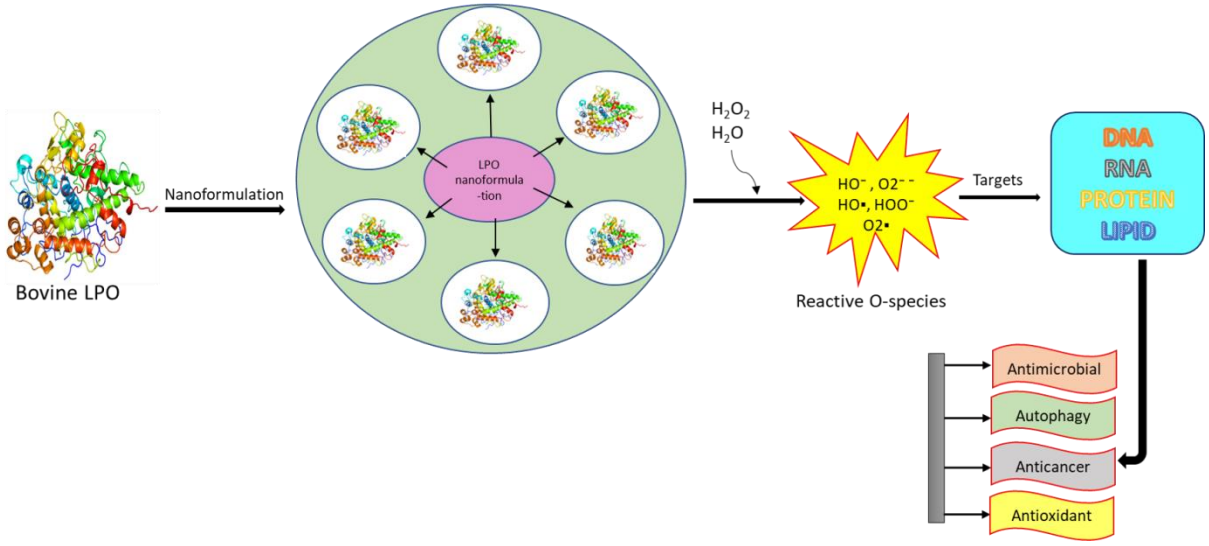


Figure S2. Diagrammatic representation of multiple applications of LPO nano formulation.

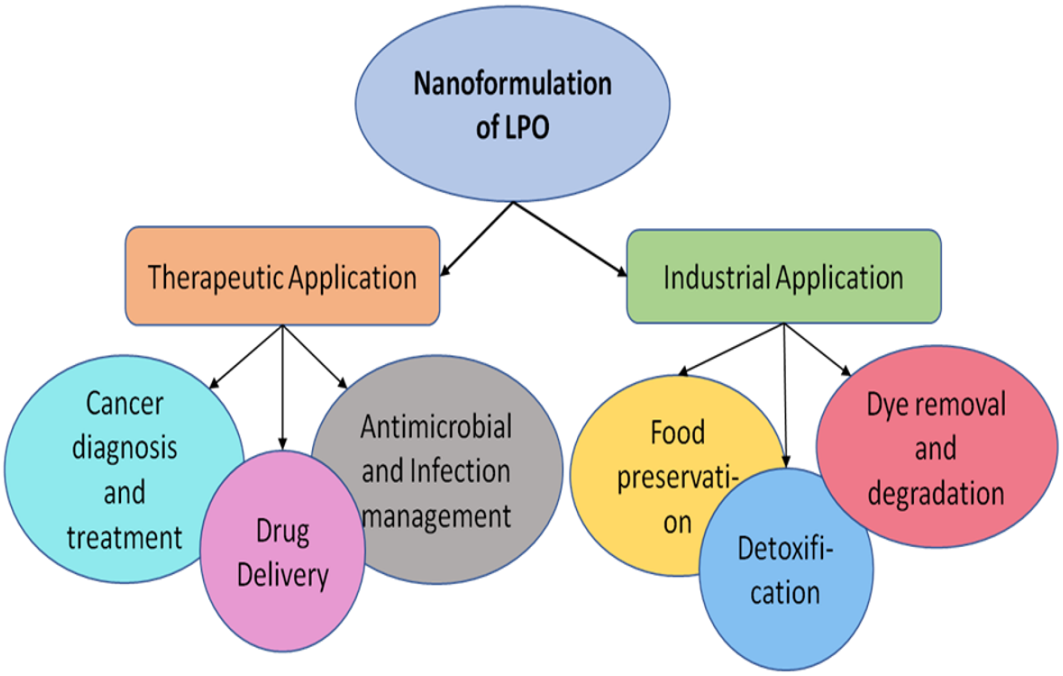


Figure S3. (A) Optical density measurement of purified LPO at 280 nm, the concentration of the protein is shown as 20.99 mg/ml (B) SDS-PAGE showing the bands of purified bovine LPO along with standard molecular weight markers.

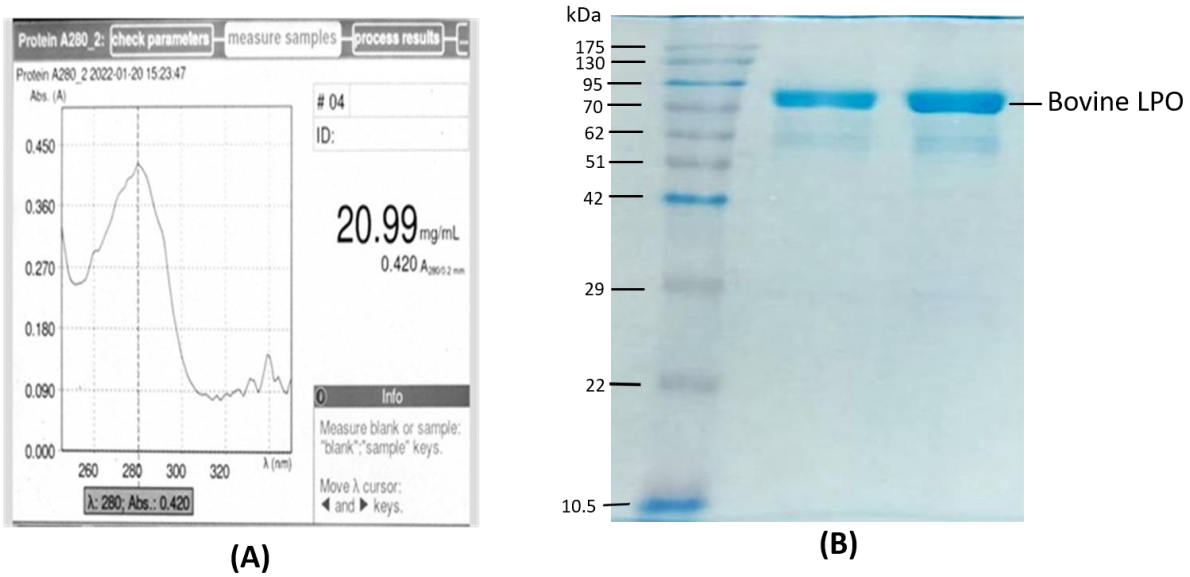
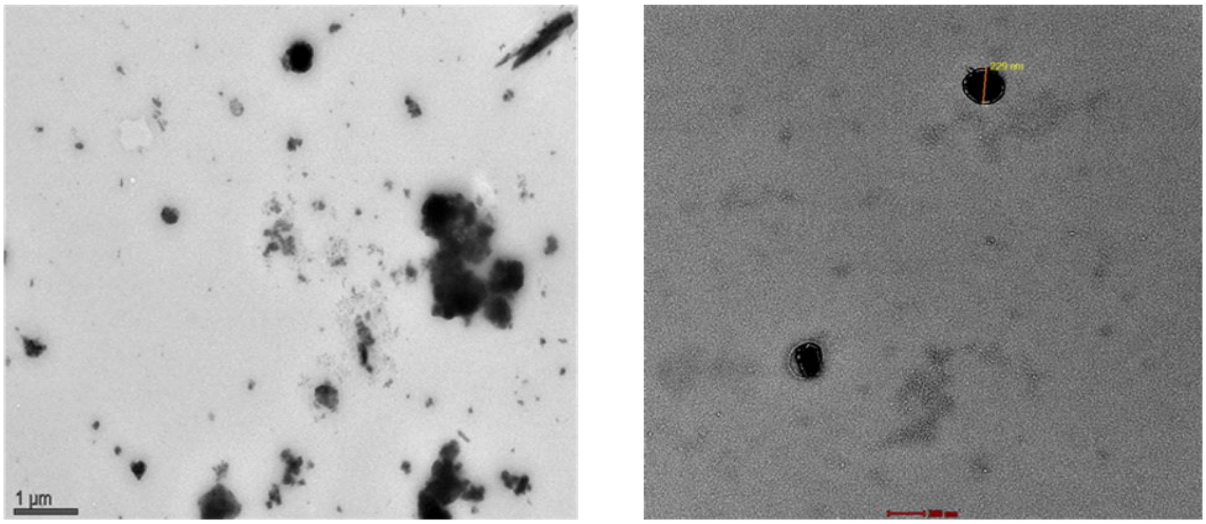
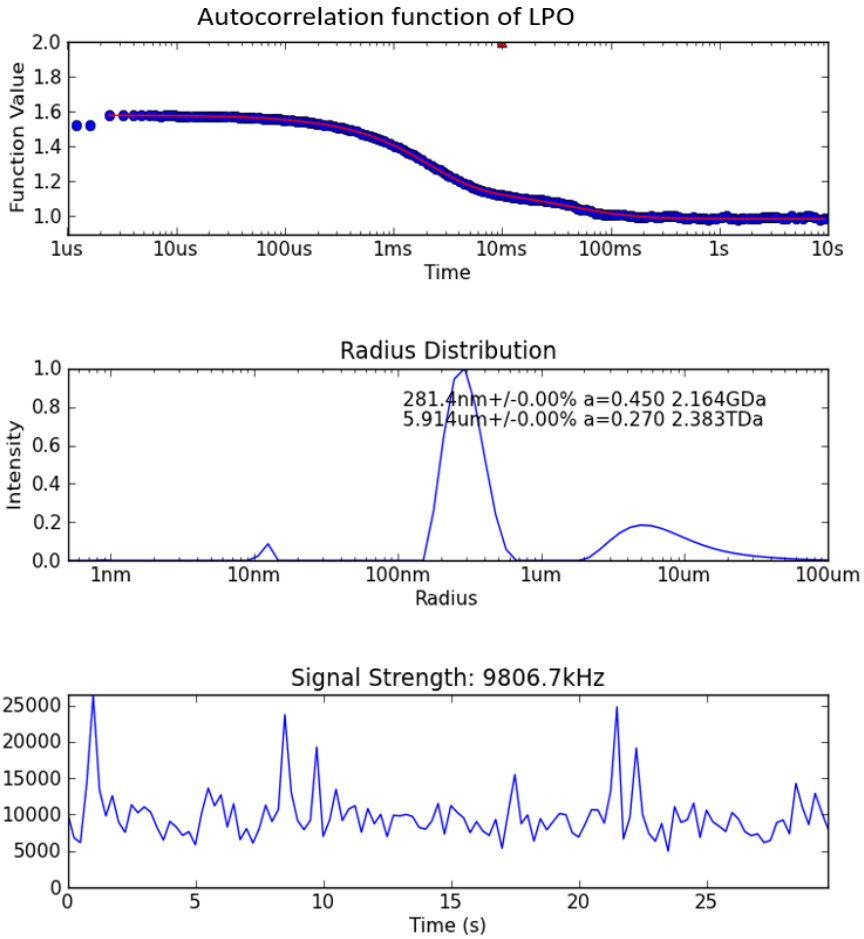


Figure S4. (A) Transmission electron micrographs (TEM) of LPO nano formulation. The image showed bovine LPO nanoparticles prepared at pH 7, holding time 20 min at 75 °C.

The larger particle size was found 1µm while smallest particle size was 229 nm. (B) Dynamic light scattering (DLS) of LPO nanoparticles showing particle diameter of 281.4 nm.



(A)


Autocorrelation function of LPO
Function Value
2.0
1.8
1.6
1.4
1.2
1.0
1us
10us
100us
1ms
10ms
100ms
1s
10s
Time
Radius Distribution
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
281.4nm+/-0.00% a=0.450 2.164GDa
5.914um+/-0.00% a=0.270 2.383TDa
1nm
10nm
100nm
1um
10um
100um
Radius
Signal Strength: 9806.7kHz
Count Rate (kHz)
25000
20000
15000
10000
5000
0
0
5
10
15
20
25
Time (s)

(B)

Figure S5. Diagram showing the bacterial growth inhibition zones using agar disc diffusion method of (A) *Staphylococcus aureus* (B) *Pseudomonas aeruginosa* (C) *E. coli* (D) *Shigella flexneri*.