



Structural-functional characterization of recombinant Apolipoprotein A-I from *Labeo rohita* demonstrates heat-resistant antimicrobial activity

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Received: 16 August 2019 / Revised: 9 October 2019 / Accepted: 19 October 2019 / Published online: 16 November 2019
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Abstract

Apolipoprotein A-I is an anti-inflammatory, antioxidative, cardioprotective, anti-tumorigenic, and anti-diabetic in mammals. Apolipoprotein A-I also regulates innate immune defense mechanisms in vertebrates and invertebrates. Apolipoproteins A-I from mammals and several teleosts display antibacterial activities against Gram negative and Gram positive bacteria. The present study describes strategies to obtain high amounts of soluble purified recombinant Apolipoprotein A-I of *Labeo rohita*, an Indian major carp (rLrApoA-I). The study also reports its detailed structural and functional characterization i.e. antimicrobial activity against a number of important marine and fresh water bacterial pathogens. The rLrApoA-I was expressed in *Escherichia coli* BL21(DE3) pLysS expression host as a soluble protein under optimized conditions. The yield of purified rLrApoA-I was ~ 75 mg/L from soluble fraction using metal ion affinity chromatography. The authenticity of the rLrApoA-I was confirmed by MALDI-TOF-MS analysis. The secondary structure analysis showed rLrApoA-I to be predominantly alpha helical, an evolutionary conserved characteristic across mammals and teleosts. The purified rLrApoA-I exhibited antimicrobial activity as evident from inhibition of growth of a number of bacteria namely *Aeromonas hydrophila*, *A. liquefaciens*, *A. culicicola*, *A. sobria*, *Vibrio harveyi*, *V. parahaemolyticus* and *Edwardsiella tarda* in a dose-dependent manner. Minimum bactericidal concentration for *A. liquefaciens*, *A. culicicola*, and *A. sobria*, was determined to be 25 µg/ml or 0.81 µM whereas for *A. hydrophila*, *E. tarda*, *V. parahaemolyticus* and *V. harveyi*, it was determined to be 100 µg/ml or 3.23 µM. These data strongly suggest that recombinant ApoA-I from *Labeo rohita* could play a role in primary defense against fish pathogen. Further, at temperature ≥ 55 °C, though a loss in secondary structure was observed, no effect on its antibacterial activity was observed. This is of significance as the antibacterial activity is not likely to be lost even if the protein is subjected to high temperatures during transport.

Keywords Apolipoprotein A-I · Antimicrobial activity · Gram-negative bacteria · *Aeromonas species* · *Vibrio species* and *Edwardsiella tarda* · *Labeo rohita*

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00253-019-10204-7>) contains supplementary material, which is available to authorized users.

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Introduction

Pathogenic infections are a major concern in the aquaculture industry leading to huge economic losses, bacterial infections like vibriosis, edwardsiellosis, and aeromoniasis being a major contributor. Numerous *Vibrio* species such as *Vibrio harveyi* and *Vibrio parahaemolyticus* are accounted for causing vibriosis, the most prevalent infection in halophilic environment affecting wide range of organisms such as teleosts and marine invertebrates. Infection in marine fishes such as crab, shrimp, shellfish, lobster, and oysters is reported to show acute hepatopancreatic necrosis syndrome and even death (Kim and Bang 2008). Edwardsiellosis, caused by *Edwardsiella tarda*, a Gram-negative and facultative anaerobic bacterium, has been reported worldwide in economically important fresh water, brackish

water, and marine fish species (Srinivasa Rao et al. 2001). The pathologic symptoms of enterohemorrhagic septicemia caused by *E. tarda* are commonly observed in different species of diseased fish (Benli and Yildiz 2004; Abraham et al. 2015). Abscesses, inflammation, and cutaneous ulcerations have been observed in channel catfish and striped mullet infected with *E. tarda* (Park et al. 2012). Yet another bacterial genus that results in severe economic losses to aquaculture industry is *Aeromonas*. The bacterium is inhabitant of the gastrointestinal tract and causes motile *Aeromonas* septicemia (MAS), hemorrhagic septicemia, ulcerative or red-sore disease, especially under stress conditions (Piotrowska and Popowska 2014). All the three bacterial infections spread very fast in contained culture and result in heavy mortality of fish in culture.

Thus, these bacterial infections afflict the aquaculture industry and are generally treated with antibiotics. However, antibiotic treatment is undesirable for food fishes as it leads to accumulation of the drug in fish and subsequently in the food chain. Also, bacteria often develop drug resistance upon continuous exposure to the antibiotics. Lack of any current commercial subunit vaccine further hinders successful treatment and eradication of these bacterial pathogens (Harikrishnan et al. 2003). Alternative strategies that would modulate the innate immunity of the fish would be an effective way to control infection. First line of defense plays an essential role in defending the host from a bacterial infection. Although the innate immunity is not specific against the attacking pathogen, however, it is crucially relevant in lower vertebrates and teleost fishes like *L. rohita*, wherein continued exposure to the pathogens in water poses an imminent threat to their survival. Antimicrobial peptides/proteins are produced by almost all species of life as an essential part of innate immunity. These possess broad-spectrum bactericidal activity and low propensity for resistance development. However, purification of these peptides from natural sources is time consuming and not economically viable which would also be accompanied by the exhaustion of the native source. Obtaining antimicrobial peptides using synthetic chemistry is a cumbersome process and has a limitation of length for accurate synthesis. These barriers can be overcome by the new generation of recombinant antimicrobial peptides which is cost effective and gives rise to more homogenous and consistent preparations (Marr et al. 2006).

The lipoproteins present in the plasma membrane are complex moieties of various lipid and protein components. These are classified into four major groups on the basis of lipid and apolipoproteins ratio, namely chylomicrons, very low-density lipoprotein, low-density lipoprotein, and high-density lipoprotein (Kondo et al. 2005). In vertebrates, exchangeable apolipoproteins comprise ApoA, ApoC, and ApoE characterized by low molecular weight and repeated amphipathic alpha helix regions for reversible lipoprotein binding, whereas non-exchangeable apolipoproteins comprise ApoB characterized

by amphipathic beta strand domain for irreversible lipid binding (Segrest et al. 1998; Saito et al. 2004). In addition, atypical apolipoproteins including ApoD, ApoF, ApoH, ApoI, ApoM, ApoN, and ApoO classes also play significant role in lipid metabolism (Page et al. 2001).

Apolipoprotein A-I/ApoA-I is the major component of high-density lipoproteins (HDL) also called “good cholesterol” (Oram 2003). It belongs to flexible exchangeable superfamily so it can exist in lipid-free, lipid-rich, and lipid-poor states (Beck et al. 2013). It has central role in reverse cholesterol transport pathway, activator of lecithin:cholesterol acyl transferase, and therefore exhibits atheroprotective activity. The risk of coronary artery disease is proportionate to the levels of ApoA-I, the risk being amplified in patients suffering from any microbial infection and has been proposed to be a biomarker for coronary artery disease (Rahim et al. 2016). Apart from crucial cardioprotection, its diverse protective and defensive roles have been well elucidated (Oram 2003; Chroni et al. 2003). HDL is an important part of innate immune system, plays critical role in fighting pathogens, and displays extensive antiviral activity by preventing virus penetration (Singh et al. 1999). HDL has been reported to modulate protection against trypanosomes and Gram-negative bacteria (Hubsch et al. 1995; Perez-Morga et al. 2005). It is capable of neutralizing bacterial lipopolysaccharide (LPS) and inhibits inflammatory cytokines (Ulevitch et al. 1981). Antimicrobial activity has been demonstrated for several mammalian and teleostean ApoA-I. Human ApoA-I has been shown to possess antiviral activity against herpes simplex virus, human immunodeficiency virus and xenotropic virus, and bactericidal activity against *Yersinia enterocolitica* (Srinivas et al. 1991; Wu et al. 2002; Alonso-Villaverde et al. 2003; Biedzka-Sarek et al. 2011). Like mammalian ApoA-I, ApoA-I from different fish species such as Carp (Concha et al. 2004; Dietrich et al. 2014), rainbow trout (Villaruel et al. 2007), striped bass (Johnston et al. 2008), cat fish (Pridgeon and Klesius 2013), Hong Kong grouper (Qu et al. 2014), and amphioxus (Wang et al. 2019) has been shown to confer antibacterial activity against selected bacteria. Ontogenic expression analysis of *Labeo rohita* ApoA-I (*LrApoA-I*) transcript has been reported post different infection conditions, and a significant upregulation of ApoA-I transcript post ectoparasite *Argulus siamensis* has been shown earlier (Mohapatra et al. 2016). Though this study made a statement about the minimum bactericidal concentration of *LrApoA-I* against *A. hydrophila* and *E. tarda*, its structural-functional characterization and detailed analysis of antibacterial activity against different marine and other freshwater bacteria have not been reported. ApoA-I is rich in hydrophobic amino acid residues (Lyssenko et al. 2012; Nagao et al. 2014). Recombinant expression of hydrophobic proteins using bacterial system often results in their expression as inclusion bodies with minimal biological activity even after refolding. Therefore, the current study was undertaken to

develop strategies that would result in soluble expression of bioactive recombinant ApoA-I of *L. rohita* in *E. coli*, to assess its antibacterial potential against a number of both marine and freshwater bacteria that infect fish, and to understand its structure-function relationships.

Materials and methods

Bacterial strains, vectors, and chemicals

Aeromonas hydrophila EUS112 (MTCC#12301) was kindly provided by Dr. I. Karunasagar, College of Fisheries, Mangalore (India) and all other bacterial strains, namely *A. culicicola* (MTCC#3249), *A. liquefaciens* (MTCC#2654), *A. sobria* (MTCC#1608), *Vibrio parahaemolyticus* (MTCC#451), *V. harveyi* (MTCC#7954), *Edwardsiella tarda* (MTCC#2400), *Bacillus subtilis* (MTCC#441), and *Staphylococcus aureus* (MTCC#740), were procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. *Escherichia coli* DH5 α and BL21 (λ DE3) pLysS host strains for cloning and expression respectively were obtained from GIBCO-URL, USA. Expression plasmid vectors pET22b+ and pET28a+ were obtained from Novagen, USA. Constituents for bacterial culture medium were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All the other reagents used in the study were of molecular biology grade and were obtained either from Sisco Research Laboratory, India, or Sigma-Aldrich Chemical Co., USA,

Generation of Apolipoprotein A-I expression construct

Synthetic *L. rohita* ApoA-I gene construct, without the signal sequence, codon optimized for expression in *E. coli*, was designed based on complete cds of ApoA-I mRNA (GenBank Acc. no.KC934748.1). The sequence of the synthetic *LrApoA-I* gene has been submitted to GenBank (Accession no.MK370726.1). The synthetic ApoA-I gene (729 bp), cloned in expression vectors pET22b+ (Novagen, USA) at *Nde* I *Xho* I sites at the 5' and 3' ends respectively, was obtained from Genscript, USA. The resultant construct was designated as pET22.*LrApoA-I*.

Expression of ApolipoproteinA-I in *E. coli*

Plasmid pET22.*LrApoA-I* clone was transformed into *E. coli* BL21(λ DE3) pLysS cells. The transformants were selected on LB-agar plates containing ampicillin (100 μ g/ml) and analyzed for expression essentially as described earlier (Karan et al. 2016). However, the expression level was negligible and could not be visualized by Commassie Brillinat Blue

staining. Therefore, the synthetic ApoA-I gene was excised out from pET22.*LrApoA-I* by *Nde*I and *Xho*I digestion and subcloned into pET28a+. The transformants were selected on kanamycin (50 μ g/ml)-LB agar plates and designated as pET28.*LrApoA-I*. The recombinant plasmid was transformed into *E. coli* BL21(λ DE3) pLysS and induced for expression as described earlier (Karan et al. 2016). Briefly, the transformed *E. coli* BL21(λ DE3) pLysS cells harboring pET28.*LrApoA-I* were grown until the log phase culture absorbance at 600 nm reached 0.6, following which the *E. coli* cells were induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) and allowed to grow further for 4 h at 37 °C. The cells were harvested by centrifugation at 3000 \times g for 5 min at 4 °C, re-suspended in lysis buffer (10 mM Tris-HCl, pH 7.5) and lysed by sonication as described earlier (Solanki et al. 2017). Uninduced and induced cell lysates were analyzed by SDS-PAGE (12%) for expression analysis. To assess the localization of expression, the cell lysates were centrifuged at 12,000 \times g for 20 min at 4 °C to separate the soluble proteins from insoluble fraction and the subcellular localization of r*LrApoA-I* was analyzed by SDS-PAGE (12%). To obtain maximum expression of soluble protein, the cells were induced at different temperatures and for different time points at the optimum temperature as indicated in the legends to the respective figure.

Equal protein concentrations were loaded for uninduced, induced cell lysates, soluble, and insoluble cellular fractions using Pierce BCA protein estimation kit (Thermo Fischer Scientific, USA).

Western blot analysis

Western blot analysis using anti-His monoclonal antibody was performed essentially as described earlier (Kaushik et al. 2018). The r*LrApoA-I* was resolved on the SDS-PAGE (12%) and transferred onto the nitrocellulose membrane using transfer buffer (25 mM Tris-HCl, pH 7.6, 192 mM glycine with 20% methanol) The membrane was then treated with blocking solution phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 5% non-fat milk (Difco Laboratories Inc, USA) overnight at 4 °C. This was followed by incubation with primary anti-His monoclonal antibody (Sigma-Aldrich Chemical Co., USA) diluted in PBST (1:3000) for 1 h at room temperature (RT), followed by three PBST washes of 10 min each. Secondary antibody (anti-mouse HRP, Sigma-Aldrich Chemical Co., USA) was then added (1:5000 in PBST) and incubated for 1 h at RT. After washing, the blot was extensively with PBST as described above, the blot was developed using Pierce ECL Western blotting substrate (Thermo Scientific, USA). The image was acquired in a Biospectrum 500 Imaging system (UVP, Cambridge UK).

Purification of rLrApoA-I from soluble fraction

The recombinant ApoA-I of *L. rohita* (rLrApoA-I) was purified using Hispur cobalt resin (Thermo Fischer Scientific, USA). The soluble rLrApoA-I failed to bind to the resin in its native state, due to inaccessibility of the histidine tag. Therefore, the soluble fraction was supplemented with urea (4 M) for partial unfolding and kept for binding with the resin at 25 °C for 2 h with end to end shaking. The slurry was centrifuged at 1200×g in a swinging bucket rotor for 2 min. The flow through was discarded and non-specific unbound proteins were removed by washing the resin twice with the wash buffer (10 mM Tris-HCl, pH 7.5). The rLrApoA-I was eluted using elution buffer (10 mM Tris-HCl buffer, pH 7.5, and 100 mM imidazole). Different fractions were analyzed on SDS-PAGE (12%). The fractions showing the rLrApoA-I presence were pooled together and dialysed against 10 mM Tris-HCl buffer (pH 7.5) using 3.5-kDa dialysis membrane (Spectrum Laboratories, USA). The rLrApoA-I was then concentrated using Amicon ultra-centrifugal device (Millipore, USA) and stored at –20 °C in small aliquots. Protein concentration of the purified rLrApoA-I was estimated using BCA protein estimation kit as per the manufacturer's recommendation. Commercial services of Sandor Proteomics, Hyderabad were availed to carry out MALDI-TOF-MS of the rLrApoA-I to confirm the authenticity of the protein produced in *E. coli*.

Circular dichroism spectroscopy

The secondary structure of the rLrApoA-I (0.2 mg/ml in 10 mM Tris-HCl, pH 7.5) at 25 °C was measured by circular dichroism (CD) spectroscopy in a Jasco model J-815 spectropolarimeter fitted with a temperature-regulated cuvette chamber spectropolarimeter as described by Yadav et al. (2016). Briefly, ten successive spectra were accumulated in the far UV region (200 to 250 nm) at a scanning speed of 50 nm/min in a cell with path length of 0.1 cm. The spectra were averaged followed by baseline correction. Mean residue weight ellipticities were expressed as degree × cm² × dmol⁻¹. Secondary structure contents of the folded rLrApoA-I from the CD measurements were calculated using the K2D3 software (<http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/>). In order to assess the effect of temperature on the secondary structure of the rLrApoA-I, the rLrApoA-I was incubated at different temperatures (25 °C, 35 °C, 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C) for 30 min, followed by far-UV circular dichroism spectroscopy.

Fluorescence spectroscopy

To assess the tertiary structure of the rLrApoA-I, fluorescence spectroscopy analysis of the rLrApoA-I (0.2 mg/ml in 10 mM Tris-HCl, pH 7.5) was carried out using a spectrofluorimeter

fitted with a Peltier temperature controller (Cary Eclipse, Varian Optical Spectroscopy Instruments, USA) with excitation at 280 nm for tyrosine and tryptophan residues, and 295 nm for mainly tryptophan residues (slit width 10). Emission spectra were monitored in the wavelength range of 300 nm and 500 nm (slit width 10) at a scan speed of 30 nm/min. The height at maximal emission (emission maxima) was measured. To assess the effect of temperature on the tertiary structure of the rLrApoA-I, fluorescence spectra were recorded at different temperatures. Temperature kinetics of rLrApoA-I unfolding was analyzed by fluorescence spectroscopy of the protein (excitation at 280 nm, emission between 300 and 500 nm) incubated at different temperatures for 30 min. The kinetics of unfolding is represented by plotting peak fluorescence intensity at 334 nm versus incubation temperature.

Antimicrobial activity of recombinant Apolipoprotein A-I (rLrApoA-I)

The antimicrobial activity assay of rLrApoA-I was performed as described earlier (Johnston et al. 2008; Verma et al. 2018) against the following bacterial species: *A. hydrophila*, *A. liquefaciens*, *A. sobria*, *A. culicicola*, *B. subtilis*, *E. coli*, *S. aureus*, *V. harveyi*, and *V. parahaemolyticus*. A single colony of different bacteria was inoculated in Luria Bertani broth and allowed to grow overnight. The bacterial culture was then diluted in LB to a final colony count of 1 × 10⁵ cfu/ml. Bacterial culture (1 × 10⁴ cfu in 100 μL, in triplicate) was aliquoted in 96-well plate. The rLrApoA-I was filter sterilized (0.22 μm) and serially diluted in culture medium. One hundred microliters of different dilutions of the rLrApoA-I was then added to the bacterial culture in 96-well plate to attain a final concentration of 0 to 1 mg/ml to determine minimal bactericidal concentrations (MBC). The bacteria were then allowed to grow for 12 h in a plate shaker at 37 °C. Bacterial growth was monitored by measuring the absorbance at 600 nm at every 2 h. Effect of temperature on biological activity of rLrApoA-I was studied by heating rLrApoA-I for 1 h at varying temperature prior to addition with the bacterial culture.

Disk diffusion assay for determination of zone of inhibition by rLrApoA-I

The overnight grown *A. hydrophila*, *A. liquefaciens*, *A. sobria*, *A. culicicola*, *V. harveyi*, *V. parahaemolyticus*, and *E. tarda* culture were spread on LB agar plate to obtain bacterial lawn. Different dilutions of rLrApoA-I were prepared in PBS so as to maintain the application volume constant. Sterile disks (6 mM, HiMedia Laboratories Pvt. Ltd. India) impregnated with different concentrations of rLrApoA-I (0 μg, 25 μg, 50 μg, 100 μg in a fixed volume of 10 μl) was pressed aseptically

onto the inoculated agar plate to ensure contact. The bacteria were then allowed to grow at 25 °C for 16 h. To assess the effect of temperature on antibacterial activity of *rLrApoA-I*, the *rLrApoA-I* was incubated for 1 h at varying temperature prior to use in disk diffusion assay.

Statistical analysis

The significance level of the change (*p* value) brought about by the *rLrApoA-I* was analyzed using two-way analysis of variance (ANOVA) method. For this purpose, GraphPad Prism 7 software (Chicago, IL, USA) was used.

Gene sequences

The nucleotide sequence of the codon-biased synthetic *ApoA-I* gene of *L. rohita* has been submitted to NCBI GenBank (Accession no. MK370726.1)

Results

Cloning and Expression of ApolipoproteinA-I

Synthetic codon-biased *rLrApoA-I* gene cloned into pET22b+ failed to express (data not shown). Therefore, subcloning of the 729 bp *NdeI*–*XhoI* fragment encoding the *ApoA-I* gene, excised from the pET22.*LrApoA-I*, was done in plasmid pET28a+ digested with the same enzymes and transformed into *E. coli* BL21 (λ DE3) pLysS cells for expression.

In order to get expression of the recombinant protein, we carried out induction at different temperatures and analyzed for expression and localization of the *rLrApoA-I*. As shown in Fig. 1a, no expression was observed at 15 °C. SDS-PAGE analysis of the uninduced and induced cell lysates of cultures grown at different temperature showed the presence of *rLrApoA-I* with an apparent molecular weight of ~ 31 kDa only in the induced cell lysate (Fig. 1a) at other temperatures indicating tight regulation of expression. Maximum expression was obtained in the culture induced at 37 °C.

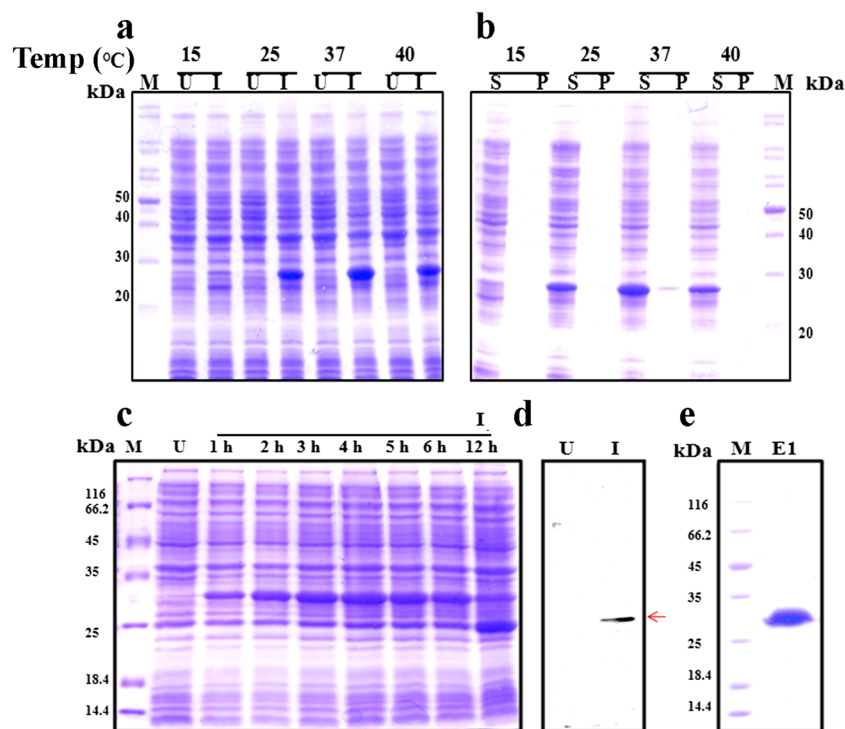


Fig. 1 Expression analysis of recombinant ApoA-I in *E. coli* BL21(λ DE3) pLysS cells harboring pET28.*ApoA-I*. **a** Effect of temperature on *rLrApoA-I* expression. SDS-PAGE (12%) analysis of the cell lysates induced at different temperatures (15 °C, 25 °C, 37 °C, 40 °C). U and I indicate the uninduced and induced cell lysates at the respective temperature. **b** Localization of expression of the *rLrApoA-I* induced at different temperatures. SDS-PAGE (12%) analysis of the soluble (S) and insoluble (I) fractions of the cell lysates of *E. coli* BL21(λ DE3) pLysS cells harboring pET28.*ApoA-I* induced at different temperatures. **c** Time kinetics of *rLrApoA-I* expression. SDS-PAGE (12%) analysis of *E. coli*

BL21(λ DE3) pLysS cells harboring pET28.*ApoA-I* induced at 37 °C for different time periods (1 to 12 h). U indicates the uninduced cell lysates. Arrow points to the *rLrApoA-I* band. **d** Western blot analysis of the *rLrApoA-I* using anti-His antibody. U and I indicate the uninduced and induced cell lysates of *E. coli* BL21(λ DE3) pLysS cells harboring pET28.*ApoA-I*. The arrow points to the *rLrApoA-I* protein of ~ 31 kDa detected only in the induced cell lysates. **e** The figure shows SDS-PAGE (12%) analysis of the *rLrApoA-I* purified using metal affinity chromatography. M in all the panels denote migration of protein molecular weight markers

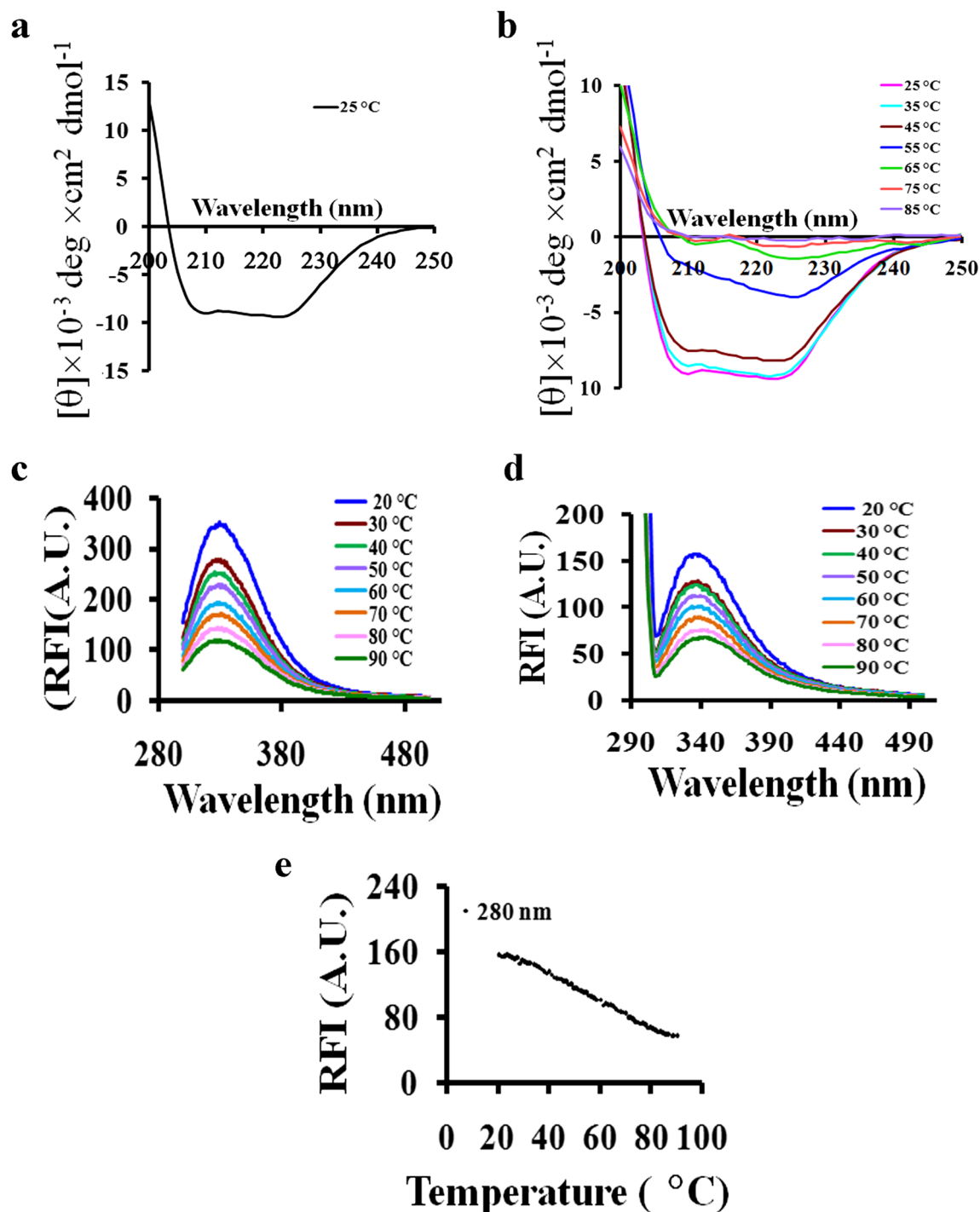


Fig. 2 Structural analysis of the *rLrApoA-I*. **a** Circular dichroism (CD) spectrum of the soluble ApoA-I in the far-UV regions (200–250 nm) at room temperature (25 °C). Mean residue mass ellipticity $[\theta]$ values are shown as degree $\times \text{cm}^2 \text{ dmol}^{-1}$. **b** Effect of temperature on the secondary structure of the *rLrApoA-I*. The *rLrApoA-I* was incubated at the indicated temperatures between 25 and 85 °C for 30 min, followed by CD spectral analysis in the far-UV regions. Mean residue mass ellipticity $[\theta]$ values are shown as degree $\times \text{cm}^2 \text{ dmol}^{-1}$. **c** Intrinsic fluorescence spectra (contributed by both tyrosine and tryptophan residues) of the *rLrApoA-I*

(excitation at 280 nm, emission between 300 and 500 nm). Kinetics of unfolding was determined by measuring intrinsic fluorescence at different temperatures. **d** Intrinsic fluorescence spectra of the tryptophan residue (Trp155) at different temperatures (excitation at 295 nm, emission between 300 and 500 nm). **e** Kinetics of temperature induced unfolding of *rLrApoA-I*. Peak fluorescence intensities obtained by analysis of intrinsic fluorescence (excitation at 280 nm, emission between 300 and 500 nm) at different temperatures are plotted against the temperature. RFI (AU) denotes relative fluorescence intensity in arbitrary units

Table 1 Secondary structural components of the *rLrApoA-I* incubated at different temperatures for 30 min prior to CD spectroscopic analysis

Incubation temperature (°C) for 30 min.	α -helix (%)	β -strand (%)	Random coil + β -turn (%)
25	79.23	1.5	19.27
35	77.54	1.55	20.91
45	68.03	2.23	29.74
55	14.75	33.31	51.94
65	2	44.08	53.92
75	0.94	44.82	54.24
85	0.89	44.88	54.23

Localization analysis revealed that all the induction temperatures, the *rLrApoA-I* expressed predominantly as soluble protein (Fig. 1b), though negligible amounts were seen in the insoluble fraction in the culture induced at 37 °C. Time kinetics of expression showed that expression of the recombinant protein increased with time until 4 h, which nearly remained constant until 6 h (Fig. 1c). These data resulted in identification of conditions that led to maximum expression of the soluble *rLrApoA-I*, and for purification of the large amounts of the protein, the culture was induced with 1 mM IPTG for 4 h at 37 °C. Western blot analysis of the cell lysates induced under these conditions using anti-His antibody confirmed the protein to be *rLrApoA-I* as a single band at the expected position appeared only in the induced cell lysates (Fig. 1d).

Purification of Apolipoprotein A-I from soluble fraction

The hexa-histidine-tagged *rLrApoA-I* was purified from the soluble fraction using Hispur cobalt resin affinity chromatography (Fig. 1e). The *rLrApoA-I* was purified to near homogeneity (> 98%) as determined by densitometric analysis of the gel with an approximate yield of ~ 75 mg/L of soluble *rLrApoA-I* at shake flask level. MALDI-TOF-MS of the purified *rLrApoA-I* further confirmed the authenticity of the purified protein (Fig. S1).

Structural characterization of the *rLrApoA-I*

Far-UV circular dichroism spectroscopy of the *rLrApoA-I* revealed it to predominantly possess α -helical structure with characteristic double minima in ellipticity at 208 nm and 222 nm (Fig. 2a). Analysis of the spectra using K2D3 software for secondary structure prediction showed the *rLrApoA-I* at ambient temperature (25 °C) to have 82.56% α -helical content, 1.24% β -strand, and 16.2% random coil. When the *rLrApoA-I* was incubated at different temperatures for 30 min, no significant change in the secondary structure was observed upto 45 °C (Fig. 2b). Incubation at 55 °C for 30 min resulted in significant loss of native tertiary structure with a shift from α -helical content to β -turns. Analysis of various secondary structure characteristics upon treatment at different temperatures is summarized

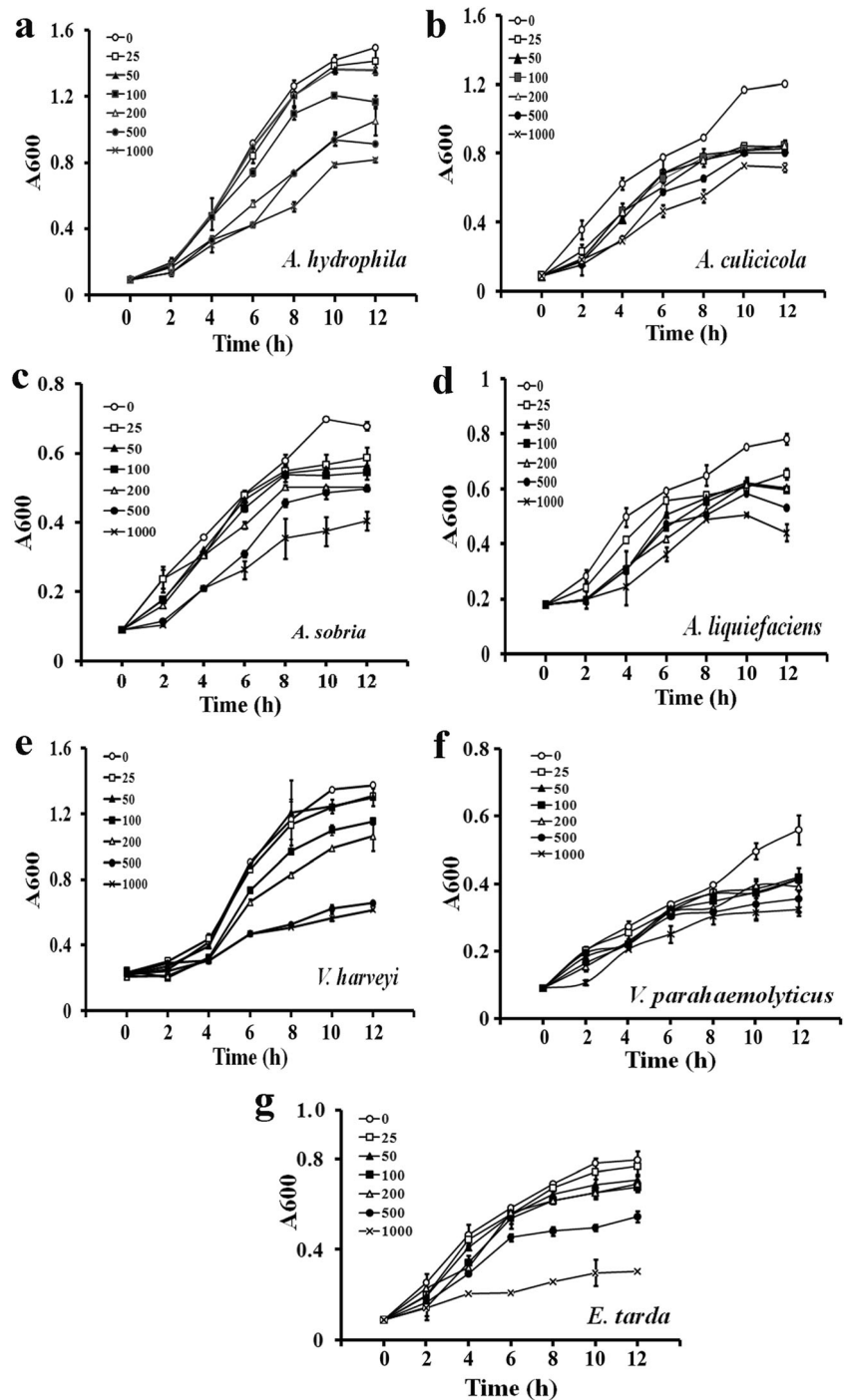
in Table 1. The T_m of *rLrApoA-I* was calculated as 50 °C. After incubation at 85 °C, the *rLrApoA-I* contained only 2.48% α -helical content and 43.27% β -strand, remainder being contributed by random coil.

Fluorescence spectroscopy of the *rLrApoA-I* was performed to analyze temperature dependence of the tertiary structure of the protein. An excitation wavelength of 280 nm was used to excite all the 8 Tyr residues and 1 Trp residues, whereas to selectively excite 1 Trp-155 at 155th position, an excitation wavelength of 295-nm wavelength was used. The *rLrApoA-I* gave emission maxima at 334 nm indicating intact tertiary structure when excited at 280 nm whereas emission maxima of 337 nm was observed when excited at 295 nm (Fig. 2 c and d, respectively). A gradual decrease in the emission maxima was observed with increase in temperature until 90 °C, indicating loss of tertiary structure. In order to study the steady state of unfolding of the *rLrApoA-I*, the protein was incubated for 30 min at each temperature followed by fluorescence spectroscopy. This analysis showed consequent decrease in fluorescence intensity at 334 nm with gradual increase of temperature at 280-nm excitation and red shift of 4 nm was observed when Trp-155 was excited at 295 nm (Fig. 2 c and d). The kinetics of the *rLrApoA-I* unfolding is evident from the gradual decrease in relative fluorescence intensity with increase in temperature (Fig. 2e).

Antimicrobial activity of recombinant Apolipoprotein A-I

In vitro antimicrobial activity assay using broth dilution assay showed that *rLrApoA-I* was able to inhibit growth of *A. hydrophila*, *A. liquefaciens*, *A. culicicola*, *A. sobria*, *V. harveyi*, *V. parahaemolyticus*, and *E. tarda* in a concentration dependent manner (Fig. 3). While *rLrApoA-I* was able to inhibit the growth of *A. culicicola* significantly ($p \leq 0.001$) at all the concentrations from 2 h onwards, it was able to inhibit growth of *A. liquefaciens* at 50 $\mu\text{g/ml}$ and higher from 2 h onwards ($p \leq 0.001$). Significant inhibition of growth could be observed from 4 h onwards for *A. hydrophila* ($p \leq 0.05$ to $p \leq 0.001$), *A. sobria* ($p \leq 0.001$), *V. harveyi* ($p \leq 0.05$ to $p \leq 0.001$) at concentrations starting from 100 $\mu\text{g/ml}$. For *V. parahaemolyticus* and *E. tarda*, significant inhibition of growth could be observed from 8 h

Fig. 3 a–f Growth profile of different bacteria (0–12 h) in the presence of different concentrations (25 to 1000 $\mu\text{g/ml}$) of the rLrApoA-I using broth dilution assay. The data represent mean \pm SD of three independent experiments performed in triplicates. **a** *Aeromonas hydrophila*, **b** *Aeromonas culicicola*, **c** *Aeromonas sobria*, **d** *Aeromonas liquefaciens*, **e** *Vibrio parahaemolyticus*, and **f** *Edwardsiella tarda*. The significance level (p values) of the change observed in growth of different bacteria treated with varying concentrations of the protein at different time intervals in comparison to the control cells treated with PBS only at the respective time point is shown in Fig. S2



onwards at concentrations 100 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$ of rLrApoA-I ($p \leq 0.001$). At 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, the rLrApoA-I was able to significantly inhibit the growth of all the test bacteria from 2 h onwards ($p \leq 0.005$ to $p \leq 0.001$). No inhibition of growth was observed for *B. subtilis*, *E. coli*, and *S. aureus* (data not shown). Percentage growth inhibition of all the bacteria at 12 h of growth period at different concentration is shown in Fig. 4. As evident, a dose-dependent increase in growth inhibition could be seen with *A. hydrophila*, *A. sobria*,

A. liquefaciens, *V. parahaemolyticus*, and *V. parahaemolyticus*. Minimum bactericidal concentration for *A. hydrophila*, *E. tarda*, *V. parahaemolyticus*, and *V. parahaemolyticus* was found to be 100 $\mu\text{g/ml}$ or 3.23 μM whereas for *A. liquefaciens*, *A. culicicola*, and *A. sobria*, minimal bactericidal concentration was found to be 25 $\mu\text{g/ml}$ or 0.81 μM . The antibacterial property of rLrApoA-I was further confirmed with an increase in zone of inhibition in the presence of rLrApoA-I on LB agar plate for *A. hydrophila*, *A. liquefaciens*, *A. culicicola*, *A. sobria*, *V. parahaemolyticus*,

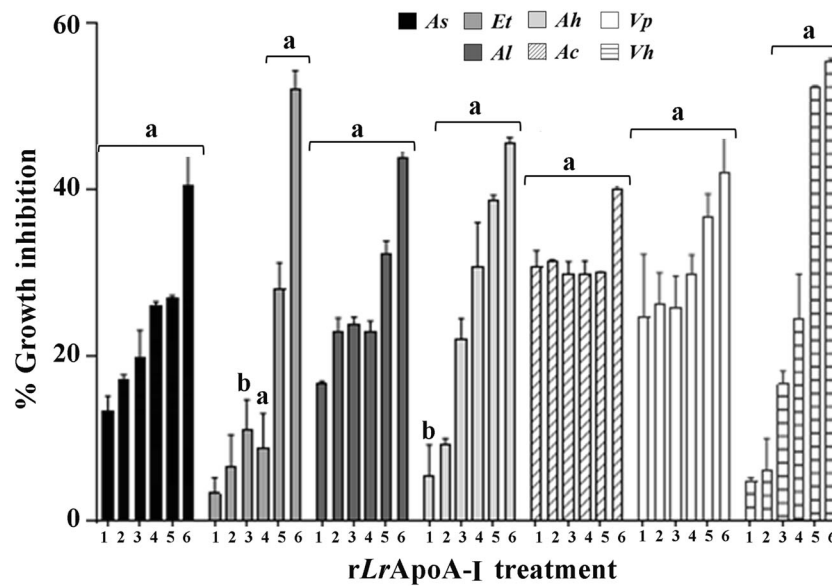


Fig. 4 Percentage growth inhibition of different bacteria at 12 h of growth at 37 °C in liquid culture in the presence of different concentrations of rLrApoA-I. *Aeromonas hydrophila* (Ah), *A. liquefaciens* (Al), *A. culicicola* (Ac), *A. sobria* (As), *Vibrio harveyi* (Vh), *V. parahaemolyticus* (Vp), and *Edwardsiella tarda* (Et). Percentage growth inhibition is calculated by taking the absorbance of untreated

cells grown at 37 °C for 12 h as 100% growth. The data represent mean ± SD of three different experiments performed in triplicate. The numbers 1, 2, 3, 4, 5, and 6 on X-axis denote rLrApoA-I concentrations at 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 500 μg/ml, and 1000 μg/ml, respectively. The letters a and b indicate *p* values ≤ 0.001 and ≤ 0.005, respectively

V. parahaemolyticus, and *E. tarda* culture. The zone of inhibition obtained with different bacteria in the presence of different concentrations of rLrApoA-I is summarized in Table 2.

Since the rLrApoA-I caused significant growth inhibition (*p* ≤ 0.001) of *A. hydrophila*, *A. culicicola*, and *V. harveyi*, these bacteria were chosen to assess the effect of temperature on the antibacterial activity of rLrApoA-I. As shown in Fig. 5a–c, there was a significant decrease (*p* ≤ 0.001) in the growth of these bacteria in the presence of rLrApoA-I. No

decrease in the growth inhibitory activity of the rLrApoA-I was observed even when it was pre-incubated at temperatures higher than the room temperature up to 85 °C prior to its activity assay for these bacteria. To assess if the antibacterial activity of the heated rLrApoA-I is due to rapid reanuration during the assay, we compared the CD spectra of the protein sample heated at 85 °C for 30 min with that of the protein sample which was heated at 85 °C for 30 min and allowed to cool down to room temperature for 1 h. As evident from Fig.

Table 2 Zone of inhibition of different bacteria at varying concentrations of rLrApoA-I, after 16 h incubation at 25 °C, employing disc diffusion assay

rLrApoA-I conc (μg)	Bacterial strain				
	<i>A. hydrophila</i>	<i>A. culicicola</i>	<i>A. liquefaciens</i>	<i>A. sobria</i>	<i>V. harveyi</i>
0	0	0	0	0	0
25	15	15	15	15	12
50	25	20	20	20	17
100	28	25	28	25	23

rLrApoA-I conc (μg)	Bacterial strain	
	<i>V. parahaemolyticus</i>	<i>E. tarda</i>
0	0	0
100	0	9
200	10	10
500	14	10

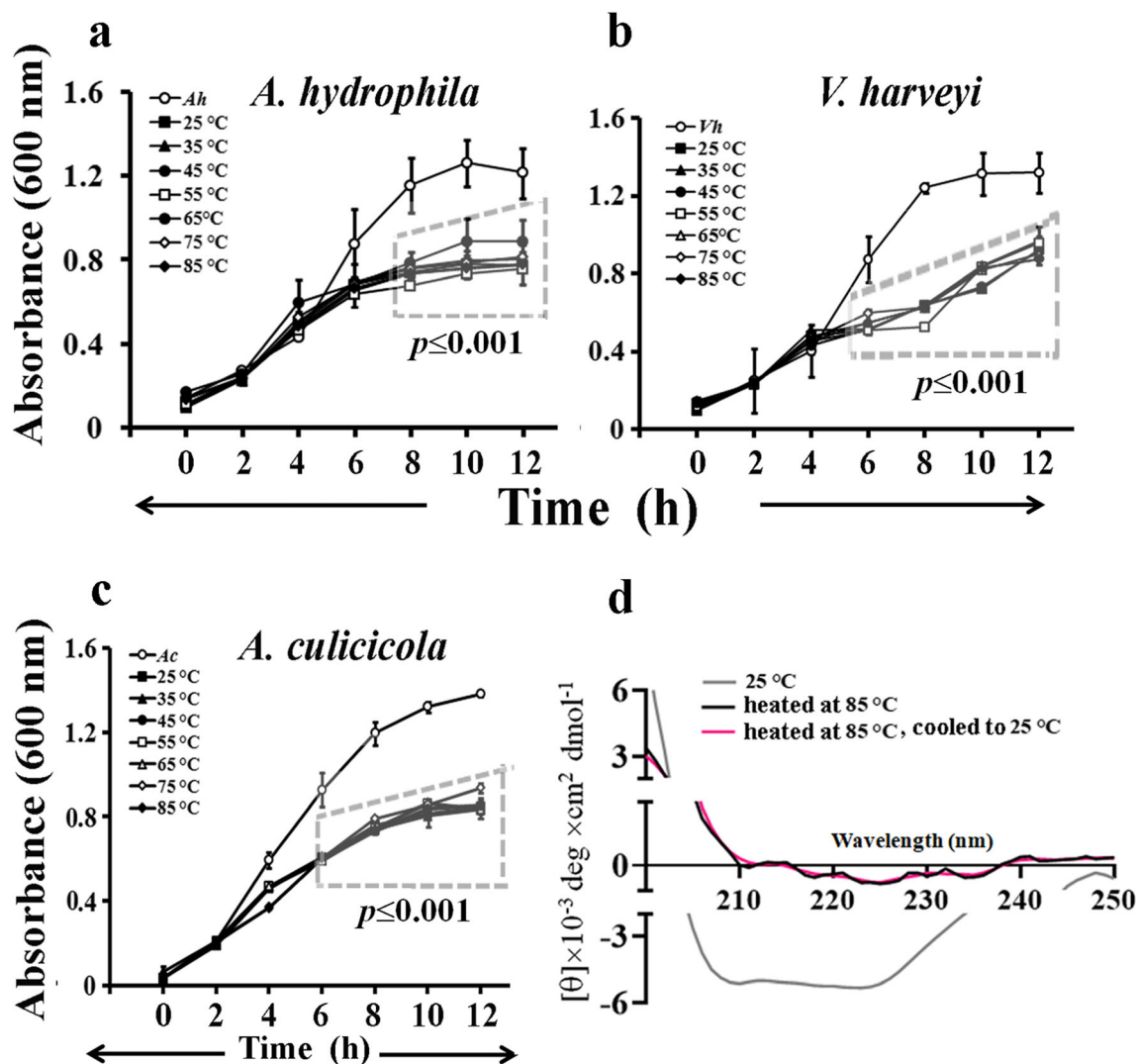


Fig. 5 Effect of temperature on growth inhibition ability of the rLrApoA-I on **a** *A. hydrophila* (*Ah*), **b** *V. harveyi* (*Vh*), and **c** *A. culicicola* (*Ac*). The rLrApoA-I was incubated at different temperatures for 1 h prior to its use in the bacterial growth curve assay at a concentration of 200 $\mu\text{g/ml}$. The *Ah*, *Vh*, and *Ac* indicate cells treated with corresponding volume of PBS. The points with significant change level of $p \leq 0.001$ with respect to PBS-

treated control cells are boxed in grey dotted line box. **d** Comparative analysis of CD spectra (in the far-UV region) of the rLrApoA-I heated to 85 °C and cooled down to room temperature with that of the rLrApoA-I heated to 85 °C. CD spectrum of the control rLrApoA-I at 25 °C is also shown. Mean residue mass ellipticity $[\theta]$ values are shown as degree $\times \text{cm}^2 \text{dmol}^{-1}$

5d, the spectra of the two samples (heated alone and cooled after heating) were identical and no renaturation of the protein was observed after cooling.

Discussion

Apolipoprotein A-I is the major protein of HDL (70%) and plays a critical role in primary immune response pathway. It has host-defensive role and fights against viral, bacterial, and parasitic infections. The mature human ApoA-I is obtained after proteolytic processing of the preproprotein as a single polypeptide chain of 243 amino acids and consists majorly of amphipathic alpha helices (Law and Brewer Jr 1984; Phillips 2013). ApoA-I has

been well studied in human and has also been identified in various mammalian and non-mammalian species rat (*Rattus rattus*), rabbit (*Oryctolagus cuniculus*), chicken (*Gallus gallus domesticus*), salmon (*Salmo salar*), cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), seabream (*Sparus aurata*), skate (*Raja erinacea*), brown bullhead (*Ameiurus nebulosus*), and sea turtle (*Chrysemys picta*) (Denslow et al. 1994; Llewellyn et al. 1998; Duggan and Callard 2001; Duggan et al. 2000; Magnadottir and Lange 2004).

Immunomodulatory role of the ApoA-I of *L. rohita* and its ontogenic expression under different infection conditions have been reported (Mohapatra et al. 2016). However, being a eukaryotic protein, the bottlenecks to produce this protein in a heterologous expression system for further analysis need to

be taken care of. In order to understand the structure function relationship of the ApoA-I of *L. rohita*, attempt to express the *L. rohita ApoA-I* gene in *E. coli* resulted in no expression. Therefore, a synthetic gene (without the signal sequence) was designed according to codon preference of *E. coli*.

It has been reported earlier that due to its amphiphilic property and instability, expression and purification of Apolipoprotein is not always easy as the protein tends to show auto-aggregation and degradation (Schmidt et al. 1997). Initial attempt to express the native *ApoA-I* gene of *L. rohita* in *E. coli* did not result in any expression (data not shown). Therefore, we chose to express a codon-biased *L. rohita ApoA-I* gene in *E. coli*. Analysis of expression showed that unlike the native *ApoA-I* gene of *L. rohita*, the codon-biased synthetic hexa-histidine-tagged *LrApoA-I* could be successfully and efficiently expressed in *E. coli*. The evident increase in the intensity of the *rLrApoA-I* protein band at the expected size in the cells harboring the codon-biased *ApoA-I* gene is much greater than the predicted theoretical increase ~ 10% to ~ 20% of total soluble protein after codon optimization which was in fact undetectable when expressed from native gene sequence (Plotkin and Kudla 2011). More than five-fold increase in yield of the human ApoA-I from optimized cDNA was obtained as compared with the original cDNA (Ryan et al. 2003). It appears that the *L. rohita's ApoA-I* gene codon preference is much far apart from *E. coli* than human ApoA-I as the codon optimization of *L. rohita ApoA-I* resulted in very good expression whereas no expression was seen with the native gene. This dramatic increase in the expression of the *rLrApoA-I* resulted in higher expression because “rare” codons were replaced with abundant codons, and the codon adaptation index (CAI) was increased using the optimized sequence. Our results are in agreement with those of Khalili and coworkers who also reported a significant increase in the expression of recombinant and biologically active human heparin-binding epidermal growth factor (HB-EGF3) through codon optimization in an *E. coli* expression system (Khalili et al. 2015). Often over-expression of the recombinant protein leads to the formation of inclusion bodies. For a protein to be biologically active, it is important that the protein is expressed in soluble form. Therefore, recombinant expression of the codon-biased *rLrApoA-I* gene was optimized to obtain maximum expression of the soluble protein by carrying out induction at different temperatures, i.e., 15 °C, 25 °C, 37 °C, and 40 °C. Analysis of soluble and insoluble fractions of the cultures induced at different temperatures clearly showed maximum expression at 37 °C in soluble fraction, though some amount of protein was also seen in insoluble fraction. Even at 40 °C, though there was a decrease in the expression, the protein mostly remained in the soluble fraction. These data show that the protein did not form aggregate even at 40 °C. These observations are in line with the stable secondary structure of the protein which did not show any significant change in the

secondary structure even when heated up to 45 °C for 30 min. Initial attempts to purify the protein using standard binding buffer (10 mM Tris-HCl buffer, pH 7.5) did not result in any binding of the *rLrApoA-I* with the resin, possibly due to the inaccessibility of the histidine tag in the soluble protein. Therefore, to make the hexa-histidine tag of the *rLrApoA-I* to the immobilized Co^{2+} , 4 M urea was included in the soluble fraction of the cell lysate, so as to allow partial unfolding of the protein. The exposed portion of hexa-histidine tag was hence made available for binding with the resin and the soluble *rLrApoA-I* was thus recovered from the soluble fraction of the induced culture using single-step purification from heterologous expression system with a yield of ~ 75 mg of the purified *rLrApoA-I* from 1 L of culture at shake flask level. This yield of soluble *rLrApoA-I* of *L. rohita* is far greater than that reported by Smith and coworkers for human ApoA-II (~ 10 mg/L) (Smith et al. 2012). The yields obtained in the present study are comparable with those reported by Ryan and his associates wherein individual mutations of the codons for proline, arginine, leucine, and one glycine were incorporated in the cloned gene converted low abundant codon to high-frequency codons (Ryan et al. 2003). MALDI-TOF-MS analysis confirmed the authenticity of the *rLrApoA-I* of *L. rohita* as the mass analysis of the peptides generated from proteolytic cleavage matched with the amino acid sequence of the theoretically generated peptides of the *L. rohita ApoA-I*.

In order to understand structure function relationship, it was important to initially know the secondary structure of the *rLrApoA-I* and any alteration that changes in temperature may induce. Secondary structure prediction of the *rLrApoA-I* using CD spectroscopy exhibited 82.56% alpha helices, which is much greater than that reported for human ApoA-I (50–57% alpha helical content). Theoretical prediction of the secondary structure from the amino acid sequence of human ApoA-I indicated presence of amphipathic helices of repeating 11 or 22 amino acids separated by proline residues (Segrest et al. 1998). The predicted secondary structure has been found to be conserved among vertebrates and teleost (Bashtovyy et al. 2011). This evolutionarily conserved structure may be responsible for the conserved defensive function of ApoA-I (Concha et al. 2004).

Temperature-induced unfolding kinetics of the purified *rLrApoA-I* of *L. rohita* was assessed by fluorescence spectroscopy. Protein sequence of mature Apolipoprotein A-I contains one phenylalanine residue, eight tyrosine residues, and one tryptophan residues with intrinsic fluorescence properties. However, for tertiary structure characterization, we measured Tyr and Trp fluorescence because their quantum yield was high enough to give a good fluorescence signal. Fluorescence spectra for both the tryptophan and tyrosine show a decrease in fluorescence with an increase in temperature from 20 to 90 °C, with a red shift in the absorbance maximum. The decrease in the fluorescence with increase in temperature thus appears to be due to some loss of

tertiary structure, and not due to the quenching of the intrinsic fluorescence with an increase in temperature. The native folded state of rLrApoA-I contained tyrosine and tryptophan residues within the core of the protein, whereas in a partially folded or unfolded state, they become exposed to solvent. In a hydrophobic environment, tyrosine and tryptophan resulted in high quantum and hence high fluorescence intensity. Thermal denaturation resulted in unfolding of the protein thus exposing the 8 Tyr and 1 Trp residues to a hydrophilic environment reflected by decreased fluorescence intensity. Thus, while the secondary structure of the rLrApoA-I remained stable until 45 °C, there was a temperature-dependent loss of tertiary structure.

ApoA-I from mammals has been demonstrated to have a variety of biological activities such as its role in cholesterol transport, lipid metabolism, and anti-atherogenic properties (Lewis and Rader 2005). Protective and immunomodulatory role of ApoA-I in mammals, lower vertebrates, and as well as in some teleost fishes has also been reported (Zamanian-Daryoush and DiDonato 2015; Mohapatra et al. 2016). Native ApoA-I isolated from different fish species such as *Cyprinus carpio* (carp), *Oncorhynchus mykiss* (rainbow trout), *Morone saxatilis* (striped bass), and *Ictalurus punctatus* (channel catfish) has been reported to possess lytic and antimicrobial activity in vitro against Gram-positive and Gram-negative bacteria (Concha et al. 2004; Johnston et al. 2008; Pridgeon and Klesius 2013). Though ApoA-I from different organisms do not share high degree of identity (Mohapatra et al. 2016), the antimicrobial function of ApoA-I is retained through evolution. This led us to assess if the recombinant ApoA-I of *L. rohita* expressed in *E. coli* retained its antimicrobial properties. Potential antibacterial effect of the rLrApoA-I against a number of important Gram-negative bacterial (both freshwater and marine) pathogens that infect both fish species and mammals was evaluated. Presence of rLrApoA-I resulted in significant reduction of bacterial growth of the selected bacteria, namely *A. hydrophila*, *A. liquefaciens*, *A. sobria*, *A. culicicola*, *V. harveyi*, *V. parahaemolyticus*, and *E. tarda*, both in liquid culture using broth dilution assay as well as on agar plates using disc diffusion assay. Thus, the rLrApoA-I of *L. rohita* expressed in heterologous expression system exhibited broad spectrum antibacterial activity. ApoA-I from other species including teleosts have been reported to inhibit growth of few bacteria (Beck et al. 2013), whereas in the present study, we report antibacterial activity of the rLrApoA-I of *L. rohita* against a number of bacteria that result in major economic losses to both freshwater and marine aquaculture. For *L. rohita* ApoA-I also, MIC concentration of ApoA-I only for *A. hydrophila* and *E. tarda* has been reported without any detailed analysis on their growth kinetics etc. The observed MIC of rLrApoA-I in the present study is in agreement with the reported MIC (100 µg/ml) of recombinant ApoA-I from channel catfish, striped bass, and Hong Kong grouper fish against a number of both Gram-positive and Gram-negative bacteria including *A. hydrophila*,

V. harveyi, and *E. tarda* (Johnston et al. 2008; Pridgeon and Klesius 2013; Qu et al. 2014; Mohapatra et al. 2016). Growth inhibitory effect of ApoA-I from amphioxus (*Branchiostoma belcheri*) against *A. hydrophila* and *V. vulnificus* at 1.47 µM and 2.15 µM, respectively has recently been reported (Wang et al. 2019). The recombinant ApoA-I from channel catfish showed MIC against *A. hydrophila* as high as 6 µM (Pridgeon and Klesius 2013). Thus, the observed MIC of rLrApoA-I falls within the range reported for ApoA-I from other species. The antibacterial activity of ApoA-I can be attributed to its amino acid composition and its structure. Human and mouse ApoA-I are composed of an N-terminal helix bundle and independently folded C-terminal domain (Koyama et al. 2009). The N- and C-terminal helical regions in the ApoA-I molecule contribute to the strong lipid-binding properties of this protein as well as the conformational stability in solution (Wang et al. 2007; Palgunachari et al. 1996). Ionic interactions also play an important role in binding interaction of ApoA-I and negatively charged membrane components of outer and inner bacterial membrane composed of LPS and phosphatidylglycerol (PG) respectively. Human ApoA-I is able to bind lipopolysaccharides present in the outer membrane of Gram-negative bacterium and has been shown to have a strong preference for bilayer vesicles consisting of phosphatidylglycerol over phosphatidylcholine (Beck et al. 2013). The C-terminal residues 220–241 play a major role in binding of ApoA-I to phospholipids and also interact with LPS (Palgunachari et al. 1996; Laccotripe et al. 1997; Henning et al. 2011). The role of lysine residues of ApoA-I and the contribution of the C-terminal domain in the interaction of ApoA-I with bacterial membrane components have also been investigated (Ajees et al. 2006; Jenssen et al. 2006; Kaonis et al. 2011). Studies suggest that alpha-helical and amphipathic content of the protein is responsible for the lytic and pore formation-mediated antimicrobial properties (Perez-Morga et al. 2005). ApoA-I recognizes *Yersinia enterocolitica* through O-antigen present on the surface and the activity of ApoA-I-mediated pathogen interaction is directly proportional to the amount of O-antigen (Biedzka-Sarek et al. 2011). Human ApoE has been reported to effectively neutralize potential lethal dose of LPS in bacterial membrane (van Oosten et al. 2001). Thus, high cationic residue content, large hydrophobic regions, and the presence of lysine residue at 223 positions the *L. rohita* ApoA-I might be playing a significant role in protection against Gram-negative bacterial pathogens.

It is of interest to note that though a loss in secondary structure of the rLrApoA-I was seen at and above 55 °C, no loss in the antibacterial activity of the rLrApoA-I against *V. harveyi*, *A. culicicola*, and *A. hydrophila* (against which the protein exhibited maximum zone of inhibition) was observed even when the protein was incubated up to 30 min at temperatures up to 85 °C. Comparative analysis of the CD spectrum of the rLrApoA-I sample which was heated to 85 °C and allowed to cool down to room temperature with the

CD spectrum of the *rLrApoA-I* heated at 85 °C only showed that there was no difference in the CD spectra of the two samples, thus ruling out the possibility of rapid renaturation during the time of the antibacterial activity assay. Our results are in agreement with the earlier report on recombinant human ApoA-I where also no renaturation was observed after cooling of the sample which was heated for only 10 min (Kim et al. 2000). This indicates that the loss in secondary and tertiary structure is not essential for antibacterial activity. Antimicrobial property of few proteins such as napin has been attributed to the permeabilization of the pathogen's membrane caused by electrostatic contacts between basic residues of the protein and acidic phospholipids present on the membrane of the pathogen (Rico et al. 1996). Amino acid sequence analysis of the codon-biased *rLrApoA-I* using Peptide hydrophobicity/hydrophilicity Analysis Tool (Peptide 2.0 Inc., available online) shows that it is highly rich in hydrophobic (41%) and basic amino acid residues (19.25%). Thus, it is likely that penetration and permeabilization of the bacterial membrane by the hydrophobic and basic amino acid residues of the *rLrApoA-I* itself result in the antibacterial activity and that the integrity of secondary and tertiary structure is not absolutely necessary for its antibacterial activity.

In conclusion, we have presented strategies to produce biologically active recombinant ApoA-I of *Labeo rohita* in large amounts, which showed broad spectrum antibacterial activity against a number of Gram-negative bacteria of both marine and freshwater environment. The structure-function analysis revealed that the protein retained its antibacterial activity even after the loss of secondary and tertiary structure. Retention of antibacterial property even when protein is subjected to higher temperatures is of significance as it overcomes the loss of biological activity for most proteins during transport. Since the ApoA-I of *L. rohita* has been shown to confer an immunomodulatory role also, the observed antibacterial activity together with its immunomodulatory role is beneficial for its use in fish cultures. However, application of any therapeutic, in this case *rLrApoA-I*, in aquaculture by injection is not practical as it is tedious and time consuming. Administration of *rLrApoA-I* to fish by immersion could also lead to its degradation. Therefore, oral administration using pH-sensitive formulation or edible nano-formulations could possibly be employed for its use in aquaculture as reported earlier (Kulkarni et al. 2014; Costa et al. 2016; Shaalan et al. 2016; Mathews et al. 2018). Further studies using site-directed mutagenesis would help identify amino acid residues of the ApoA-I critical for its antibacterial activity and may give insight into mechanism of *rLrApoA-I* action. This may lead to generation of potential therapeutic antibacterial peptides for application in aquaculture.

Acknowledgments AD acknowledges the PURSE grant from the Department of Science and Technology [SR/PURSE/Phase2/11(C)

2015], New Delhi, India, to the Jawaharlal Nehru University (JNU), New Delhi, India, and the financial support from the University with Potential for Excellence grant (UPE-II – Project ID#43) from the University Grants Commission, New Delhi to JNU, New Delhi. LCG acknowledges the Indian National Science Academy, New Delhi for Senior Scientist Fellowship SK thanks the ICMR for providing research fellowship.

Funding information The present study has been carried out with the financial support from the Department of Science and Technology PURSE grant to the JNU (SR/PURSE/Phase2/11(C) 2015) and the University with Potential for Excellence grant (UPE-II–Project ID#43) from the University Grants Commission, New Delhi, India, to the JNU.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal participants performed by any of the authors.

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