Physicochemical characterization of E. coli-derived human serum albumin and its comparison with the human plasma counterpart reveals it as a promising biosimilar

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1. Introduction

Human Serum albumin (HSA) is the most abundant protein in the blood plasma. It is a non-glycosylated multidomain heart-shaped protein which performs a wide range of important functions (Fanalia et al., 2012; Kobayashi, 2006; Peters, 1996). Because of the advantageous properties of the protein, it has immense clinical and biotechnological applications. It is administered to treat several diseases and is also used as an excipient and stabilizer of therapeutic drugs and vaccines and as a supplement of cell culture medium (Boldt, 2010; Fanalia et al., 2012; Kobayashi, 2006). The annual worldwide demand for this protein has reached 500 tons (Chen et al., 2013). Surprisingly, till date blood is the major source of the protein (Chen et al., 2013). Blood is a limited and unsafe source with the risk of its contamination by various blood-derived pathogens. Thus, there exists an indispensable need to promote non-animal derived HSA production. In the present work, we have exploited the opportunity and promoted the preparation of pathogen-free rHSA from the E. coli host which is blessed with numerous advantages like scalability, cost-effectiveness etc. Upon overcoming the difficulties to produce functional rHSA in E. coli, through engineering the biological system of protein folding in the cell, the E. coli-derived HSA has been purified to homogeneity. Its detailed physicochemical characterization has been performed, by monitoring its conformational properties, secondary and tertiary structure elements, surface properties, ligand binding properties, stability issues etc. These parameters of the recombinant protein have been compared with the naturally occurring protein from the human source. The outcome of the comparison reveals that the recombinant protein resembles exactly the same as the natural one. Hence, we propose and promote that the E. coli-derived rHSA is an ideal biosimilar for human blood plasma-derived serum albumin.

Abstract

Human serum albumin one of the most demanded proteins possess an array of clinical and biotechnological applications. Currently, the prime source for HSA production is the human blood which possesses the risk of pathogen contamination and is limited. Thus, there exists an indispensable need to promote non-animal derived HSA production. In the present work, we have exploited the opportunity and promoted the preparation of pathogen-free rHSA from the E. coli host which is blessed with numerous advantages like scalability, cost-effectiveness etc. Upon overcoming the difficulties to produce functional rHSA in E. coli, through engineering the biological system of protein folding in the cell, the E. coli-derived HSA has been purified to homogeneity. Its detailed physicochemical characterization has been performed, by monitoring its conformational properties, secondary and tertiary structure elements, surface properties, ligand binding properties, stability issues etc. These parameters of the recombinant protein have been compared with the naturally occurring protein from the human source. The outcome of the comparison reveals that the recombinant protein resembles exactly the same as the natural one. Hence, we propose and promote that the E. coli-derived rHSA is an ideal biosimilar for human blood plasma-derived serum albumin.
functional, biochemical and biophysical features of the *E. coli*-derived rHSA and compared with the commercially available plasma-derived HSA (pHSA). From our studies, the *E. coli*-derived rHSA was found to be equivalent to the pHSA in terms of functional, biochemical and biophysical properties. In addition, we have also determined the effect of the presence of fatty acids (FA) on the structural stability of the isolated *E. coli*-derived rHSA.

2. Materials and methods

2.1. Purification of *E. coli*-derived rHSA

rHSA was expressed in the soluble fraction as reported previously by our group. rHSA was purified by a two-step method which includes Ni-NTA chelating chromatography and 15ISO hydrophobic interaction chromatography. Fractions containing rHSA were identified by SDS-PAGE and were pooled. Protein was stored in 50 mM Tris, 50 mM NaCl, 1 mM DTT containing 20 mM FA (sodium caprylate). The protein concentration was determined by using an extinction coefficient of 36,500 M⁻¹ cm⁻¹ at 280 nm. Verification of the monomeric status of rHSA was performed on the native-PAGE gel electrophoresis and by carrying out size exclusion chromatography. The 20 μL of the rHSA was injected into HPLC size exclusion column (Tosho SWXL4000) and buffer used for the elution was 50 mM Tris, 50 mM NaCl, and 1 mM DTT at pH 7.4 with an elution rate of 0.5 mL/min. The FA-free form of rHSA was prepared fresh by removal of FA from the rHSA + FA fraction by following Chen protocol (Chen, 1967).

2.2. Mass spectrometric identification

Mass spectrometric identification of rHSA was done by two methods, MALDI-TOF MS and LC–MS/MS. In first set up, the isolated *E. coli*-derived rHSA was separated on 12% SDS-PAGE and protein bands were manually excised, trypsin digested and analyzed by MALDI-TOF MS. While in the second set up, the rHSA was identified by LC–MS/MS. The trypsin-digested samples were extracted and desalted. LC–MS/MS analysis was performed on Easy-nLC II (Thermo Scientific, US) connected to an ESI mass spectrometer (Bruker Daltonics). Peptides generated were analyzed using Compass HyStar 3.2, Data Analysis 4.1 and BioTools 3.2 software. For identification, an MS/MS ion search was performed using Mascot search engine (parameters: monoisotopic mass values, mass tolerance ± 0.5 Da and maximum missed cleavages = 1).

2.3. Western blotting

Purified rHSA was run on 12% SDS-PAGE and western blotting was performed as reported previously by our group (Sharma and Chaudhuri, 2017).

2.4. Esterase like activity of HSA

Esterase activity of both pHSA (5 μM) and the purified rHSA (5 μM) was carried out in a 1 mL reaction mixture at 25 °C containing 1 mM pNP (buffered with 50 mM Tris, 50 mM NaCl, and 1 mM DTT, pH 7.4) as a substrate for HSA. The formation of pNP was measured at a wavelength of 410 nm spectrophotometrically after addition of HSA and recorded every 5 s for 10 min using the kinetics/time application of DU 800 Beckman Coulter spectrophotometer. The slope which represents the rate of formation of the product (pNP) was determined for both the initial (K\text{in}) as well as for the overall (K\text{overall}) reaction from the initial and later linear region of the curve respectively as a function of time.

2.5. Ligand binding assay

A set of samples was prepared to contain 5 μM of HSA (for both rHSA and pHSA) in 50 mM Tris, 50 mM NaCl, 1 mM DTT pH 7.4 with varying naproxen concentrations (0–25 μM). The samples were incubated for 20 min at 20 °C and placed in a 1 cm quartz cuvette for measuring the fluorescence intensity of each sample when excited by using Cary Eclipse fluorimeter. Excitation was performed at 331 nm and the emission spectrum was monitored in the range of 340–440 nm with bandwidths 5 nm each. Sigma plot 11.0 software was used to fit the fluorescence curves. The fluorescence intensity at 350 nm has been plotted against the concentration of naproxen both in the presence and absence of HSA. The fluorimetric data obtained were fitted using the one-site saturation equation of the ligand binding module of Sigma Plot 11.0 to obtain the binding parameters:

\[ Y = \frac{B_{\text{max}} \cdot [L]}{K_d + [L]} \]  

Where Y represents Fraction bound; B\text{max} is the maximum specific binding; K\text{d} is the dissociation constant and L is the ligand concentration. It was based on the assumption that the intensity change results from binding to one high-affinity site and hence obeying 1:1 interaction (Lammers et al., 2013).

2.6. Thermal unfolding and refolding of HSA probed by CD

Thermal unfolding studies were carried out for the rHSA (2.5 μM) in presence and absence of FA as well as pHSA (2.5 μM) and were monitored by CD spectroscopy. The proteins were prepared in buffer 50mM Tris, 50mM NaCl, 1mM DTT at pH7.4. The samples were placed in 1mm quartz cuvette and were heated, to two different end temperatures, from 20 °C to 90 °C and from 20 °C to 55 °C with a heating rate of 1 °C/min controlled by Jasco controllable Peltier PTC-423S/15. For thermal refolding studies, the 90 °C- or 55 °C-denatured HSA (2.5 μM) solution was gradually cooled to 20 °C with the cooling rate of 1 °C/min. A continuous scan of a wavelength at 222 nm was collected for each temperature. The Mean Residual Ellipticity (MRE) values plotted against the respective temperature. The buffer was always prepared fresh at room temperature in order to minimize the heating effect on Tris. The thermal denaturation profile was analyzed when the obtained set of data was fitted in the two-state model to determine a mid-point of the unfolding transition curve using Sigma plot 11.0 software as described in the Supplementary data analysis section.

2.7. Equilibrium unfolding of HSA

Equilibrium unfolding studies were monitored by CD spectroscopy. Spectra were recorded in the far-UV region at 222 nm using JASCO J-815 CD polarimeter equipped with JASCO PTC-423S/15 using 1 mm quartz cuvette. The samples were prepared by incubating HSA (2.5 μM) with different concentration of GdnHCl (0–6 M) for one hour at 25 °C in buffer 50 mM Tris, 50 mM NaCl, and 1 mM DTT pH 7.4. Each spectrum was corrected against its blank. The MRE values were plotted against the GdnHCl concentrations to obtain an unfolding transition curve. The equilibrium unfolding of HSA was probed by intrinsic fluorescence as well. For this, the equilibrated samples in 1 cm quartz cuvette were excited at 280 nm and the emission spectra were recorded from 295 to 400 nm with a slit width of 10 nm each. Each spectrum was corrected against its blank. The relative fluorescence intensity at 340 nm and emission maximal wavelength λ\text{max} was plotted against the denaturant concentration to obtain the equilibrium unfolding transition curve.

3. Results

3.1. Purification of *E. coli*-derived rHSA

The purification of his-tagged rHSA has been carried out in a two-step manner (Fig. 1a). About 60% purity level was obtained after the first step of purification and the major low molecular weight proteins in the supernatant were removed in the flow through fraction. Whereas,
the employment of the second step helped in the removal of residual non-HSA proteins. About more than 95% of purity of E. coli-derived rHSA has been achieved in the present case. Fig. 1a shows that rHSA appears at the correct Rf in the gel corresponding to the 66 kDa band of the protein marker, identical to that of the pHSA. These results suggest that rHSA was successfully purified by the two steps of chromatographic purification.

3.2. Identification and biological characterization of E. coli-derived rHSA

3.2.1. Characterization of rHSA by mass spectrometry

The molecular weight of rHSA was found to be similar to that of pHSA (i.e. approx. 66 kDa) as observed on 12% SDS-PAGE (Fig. 1a). Further identification and characterization of purified rHSA were done using mass spectrometric analysis. Mass spectrometric analysis of E. coli-derived rHSA was done by MALDI-TOF MS and LC-MS/MS. The peptide mass fingerprinting (PMF) of in-gel trypsin digested purified rHSA was obtained by MALDI-TOF as shown in Fig. 1b. The peptides detected in MALDI TOF were searched against Swiss-Prot database that matched to HSA protein with a significant mascot search protein score of 272 represented by the inset of Fig. 1b. The tryptic-digested E. coli-derived rHSA was also characterized using ESI nLC technique in MS/MS mode. The peptides were detected first in MS mode and two significant peptides were selected separately from these peptides for further fragmentation and generation of ions in MS/MS mode. The combined search result of detected peptides in MS mode along with ions generated from two individually selected peptides against the Swiss-Prot database showed HSA protein with a significant mascot search protein score of 257. Both mass spectrometric methods, MALDI TOF MS and ESI nLC MS/MS, lead to the identification and characterization of E. coli-derived rHSA protein with a significant search score. The N- and C-terminal peptides have been detected and identified in the protein database search and thereby validating the intact N- and C-terminal of the E. coli-derived rHSA. For the characterization and identification mass spectrometry tool was exploited in the study as represented in Fig. 1b.

3.2.2. Determination of the reactivity of rHSA against specific antibodies

The protein of interest, i.e. E. coli-derived rHSA in the present case, has been determined specifically for its size and antibody reactivity (against anti-HSA antibody) by carrying out western blotting (Fig. 1c). The rHSA appeared at the same position corresponding to pHSA on the blot thereby confirming the similar size and reactivity of both the forms of HSA against the anti-HSA antibody. Thus showing the behavior of rHSA similar to that of the pHSA in terms of its reactivity and specificity against anti-HSA antibodies.

3.2.3. Determination of the monomeric nature of the protein

Native gel electrophoresis of rHSA has been carried out and compared with that of the plasma-derived form (Fig. 1c). It was found that the relative electrophoretic mobility of rHSA was similar to that of the pHSA and hence the shape and size of the recombinant protein seems to be similar to that of the plasma-derived form. The monomeric form of the protein was observed to be present predominantly in both the forms of the HSA. In case of pHSA other high molecular weight bands were also observed which corresponds to the oligomeric contamination, which is absent from the fraction of purified rHSA. The same was further confirmed by carrying out HPLC of the purified rHSA, demonstrated in Fig. 1c, which again shows the elution of the protein at the elution volume which corresponds to the presence of the monomeric form of the purified rHSA.
Structural and Functional Characterization of rHSA. A. Secondary and tertiary structure determination of rHSA and pHSA. Far-UV CD spectra of rHSA in presence and absence of FA (2.5 μM) and pHSA (2.5 μM). B. Fluorescence spectra of rHSA and pHSA. The spectra when compared with that of the plasma-derived form. E. coli-derived rHSA, when reacted with pNPA, was observed to undergo a biphasic mode of reaction as indicated by initial rate constants (Ksec) of the reaction obtained for the E. coli-derived rHSA were of the similar order of magnitude to that of the plasma-derived form.

3.3. Secondary and tertiary structure determination

The secondary structures of HSA, in the presence and absence of FA, and pHSA were compared by taking CD spectra from wavelengths 240–200 nm as shown in Fig. 2a. The α-helical content of proteins was calculated from the MRE value at 222 nm (MRE222) using the following equation (Sen et al., 2008):

$$\text{Percent } \alpha\text{-helix} = \left( \frac{\text{MRE}_{222} - 2340}{30300} \right) \times 100$$

(2)

The percentage of α-helix for rHSA in the presence and absence of FA is ∼62% and ∼50% respectively; α-helical content of pHSA is around 62%. To compare the tertiary structure of proteins, intrinsic emission fluorescence of the aromatic acid residues in a protein was exploited. HSA contains 18-tyrosine residues and 1-tryptophan residue (Kobayashi, 2006). The protein was excited at 280 nm and 295 nm to obtain intrinsic (Tyr) and intrinsic (Trp) fluorescence spectra respectively (Fig. 2a). In case of intrinsic (Tyr) fluorescence spectra, the pattern of emission of rHSA in presence and absence of FA, when compared, was found to exhibit a difference in the fluorescence intensity. FA binding leads to enhancement in the fluorescence intensity as compared to the rHSA without FA. pHSA, when excited at 280 nm, exhibited lower fluorescence intensity as compared to rHSA + FA with the red-shift in the λmax of emission. The intrinsic (Trp) fluorescence spectra obtained by excitation at 295 nm exhibited the excitation of the only tryptophan residue in a protein. The spectra when compared showed there existed change in only fluorescence intensity of rHSA + FA and pHSA without any shift in the λmax values of the two protein variants. The lowering of fluorescence emission intensity of a protein, with unaltered λmax of emission, may be due to the quenching of fluorescence through local conformational changes. However, in the presence of FA, both the rHSA and pHSA shows nearly overlapping intrinsic (Trp) fluorescence emission spectra.

3.4. Esterase activity of HSA

The esterase-like activity (Fig. 2b) of rHSA was determined and compared with that of the plasma-derived form. E. coli-derived rHSA, when reacted with pNPA, was observed to undergo a biphasic mode of reaction as indicated by initial rate constants (Ksec) and the overall rate constants (Konc) of the reaction. Both rHSA and pHSA followed the pseudo-first-order kinetics which appeared first order with an excess of HSA over the substrate which is in agreement with the previous work done on pHSA esterase activity (Salvi et al., 1997). From Fig. 2b it could be easily observed that the rate of reaction is faster initially and it further slowed down as compared to the initial rate of the reaction as represented by the change in the slope of the reaction curve. The parameters of the reaction obtained for the E. coli-derived rHSA were of the similar order of magnitude to that of the plasma-derived HSA when subjected to similar conditions as represented in Fig. 2b.

3.5. Ligand binding assay

The interaction of the naproxen with HSA (rHSA and pHSA) has been investigated using fluorescence spectroscopy as represented in Fig. 2c. Inset at the top shows fluorescence intensities of increasing concentration of naproxen in the presence and absence of 5 μM HSA. The fluorescence intensity of naproxen increases in a linear manner with increasing concentration in the absence of HSA. Whereas in the presence of HSA the initial slope of fluorescence intensity has increased in both the cases indicating the complexation of naproxen with both forms of HSA as represented in Table 1. Inset at the bottom of the graph represents the fluorescence intensity signals of naproxen in absence and presence of HSA (rHSA or pHSA) at a particular naproxen concentration (5 μM). It could be observed that fluorescence intensity signals of the naproxen increased in the presence of HSA when compared to the naproxen in the absence of HSA. Thus, both the rHSA and pHSA
Table 1
Fluorescence intensity slopes of naproxen free in solution and bound to HSA and binding constants in M$^{-1}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Slope</th>
<th>Binding constant (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen only</td>
<td>$(4.6 \pm 0.5) \times 10^6$</td>
<td>$-$</td>
</tr>
<tr>
<td>rHSA</td>
<td>$(11.4 \pm 0.6) \times 10^5$</td>
<td>$(1.1 \pm 0.4) \times 10^5$</td>
</tr>
<tr>
<td>pHSA</td>
<td>$(11.6 \pm 0.5) \times 10^5$</td>
<td>$(1.09 \pm 0.5) \times 10^5$</td>
</tr>
</tbody>
</table>

demonstrated similar fluorescence patterns when complexed with the drug naproxen. The measured fluorescence data were fitted to a one-site binding model, using sigma plot 11.0 software as represented in Fig. 2c by the dotted lines. The values of binding constants obtained are presented in Table 1. It could be observed that both rHSA and pHSA binds to the drug naproxen with the same binding affinity.

3.6. Structural and stability characterization of E. coli-derived rHSA

3.6.1. Thermal unfolding and refolding profile of rHSA

3.6.1.1. Thermal unfolding of rHSA (with- and without FA) and pHSA monitored by CD spectroscopy. Thermal unfolding profile of rHSA with- and without- FA were found to differ significantly. Fig. 3a shows that, for rHSA in absence of FA (rHSA-FA), the secondary structures had remained the same up to 25 °C, with the eventual conformational changes till 58 °C followed by a complete loss of structure after this temperature. HSA in presence of FA (rHSA + FA) on the other hand has retained the native structure with few conformational changes up to 58 °C followed by complete loss of the secondary structure at 80 °C. Similarly, pHSA, when subjected to thermal unfolding, was found to retain the secondary structure up to 50 °C followed by changes in the secondary structure and eventually complete loss at 80 °C as shown in Table 1. It could be observed that both rHSA and pHSA

The unfolding patterns and Dm values of the GdnHCl-induced unfolding of rHSA have been monitored by CD and fluorescence spectroscopy as represented in Fig. 4.

3.6.2. Chemical denaturant mediated equilibrium unfolding of rHSA

The unfolding patterns and Dm values of the GdnHCl-induced unfolding of rHSA were found to exhibit similar properties as that of rHSA as mentioned above.

3.6.2.1. Equilibrium unfolding of rHSA monitored by CD spectroscopy. In Fig. 4a rHSA- FA was observed to retain its native structure up to 0.75 M GdnHCl. Beyond this concentration, protein started losing...
native conformations and entered the unfolding transition phase. Eventually, at 2.75 M GdnHCl concentration, the maximum unfolded fraction of the protein was obtained which indicated the complete loss of the secondary structure of the protein. On the other hand, rHSA + FA shows a different unfolding profile as compared to the rHSA-FA. The rHSA + FA retained its native conformation up to 1.25 M GdnHCl and then entered into the unfolding transition zone indicated by increasing unfolded fraction. Furthermore, GdnHCl at 4.0 M concentration was able to unfold the protein completely. The unfolding transition curve was fitted into the two-state model and the Dm value was calculated to be $(2.3 \pm 0.4)$ M and $(1.7 \pm 0.5)$ M for the equilibrium unfolding of rHSA in presence and absence of FA respectively.

**3.6.2.2. Equilibrium unfolding of rHSA monitored by fluorescence spectroscopy.** Fig. 4b shows the fluorescence intensity change at 340 nm and change in \(\lambda_{max}\) in presence of increasing concentration of GdnHCl for rHSA in the absence and presence of FA. It was observed that the fluorescence intensity of rHSA-FA is higher in presence of lower concentrations of the denaturant which further increases to the maximum at 0.5 M, beyond which it decreases till 1.25 M GdnHCl with a small gradual red shift in the \(\lambda_{max}\). The initial increase in intensity is may be due to the exposure of the hidden aromatic amino acids in the core of the protein in presence of a lower concentration of the denaturant. The small shift in the \(\lambda_{max}\) is an indication of the less altered aromatic amino acid environment. Whereas a significant decrement in the fluorescence intensity and an appreciable red shift in the \(\lambda_{max}\) was observed at 3.0 M GdnHCl concentration with no further apparent changes.

For rHSA + FA an increment in the fluorescence intensity was observed, as compared to the native state, till 1.75 M GdnHCl concentration without any appreciable shift in the \(\lambda_{max}\). A gradual reduction in the fluorescence intensity and a noticeable red shift in the \(\lambda_{max}\) was observed starting from 1.75 M to 3.25 M GdnHCl. rHSA + FA showed complete unfolding at 3.25 M GdnHCl as no further changes were observed. Using a two-state model fitting (Fig. 4b), the Dm of the unfolding transition was calculated to be $(2.2 \pm 0.4)$ M and $(1.3 \pm 0.3)$ M for rHSA + FA and rHSA-FA respectively. The results obtained further validate the above-calculated Dm values from the CD data.

4. Discussion

We in the present communication have showcased *E. coli* as a successful alternate host for the production of rHSA because of the known advantages of the host. In the recent study by our lab, we had overcome the negatives associated with the *E. coli* host for rHSA production (Sharma and Chaudhuri, 2017). In the present work, we have moved a step forward in this direction by isolation and characterization of the *E. coli*-derived rHSA followed by its comparison with the commercially available plasma-derived form. Furthermore, we have also worked in the direction of understanding and improving the structural stability of the rHSA protein.

We have obtained *E. coli*-derived rHSA with more than 95% purity (Fig. 1a) which was later characterized by MALDI TOF MS and ESI nLC MS/MS (Fig. 1b). The mascot search result of peptides and ions generated by mass spectrometric analysis of rHSA was found to be identical to the native protein derived from blood, i.e. pHSA. The N- and C-terminal of the protein has been confirmed to be intact as indicated by LC-MS/MS results, ensuring that the protein is not degraded when expressed and isolated from the *E. coli* host. The physical shape and size of the protein were similar to that of pHSA protein fraction has been observed to possess significant oligomeric contamination. It has also been reported earlier that the commercially available HSA contains variable proportions of
dimer and higher order oligomers. There are factors which affect albumin aggregation during processing and its storage which are responsible for the heterogeneity of the commercially available albumin preparations (Darcel, 1987; Atmeh et al., 2007; Tankersley and Finlayson, 1980; Zini et al., 1981). The same has been reflected in our results as well and hence the presence of oligomeric form is the consequence of unpleasant pHSA storage and processing conditions which was therefore not observed in E. coli-derived rHSA fraction.

The secondary structure determined for the rHSA in presence of FA was found to be similar to that of the pHSA by Far-UV CD spectroscopy (Fig. 2a). We have investigated the tertiary structural aspects of the proteins as well based on the intrinsic fluorescence emission spectra as represented in Fig. 2a. Difference in the intrinsic (Tyr) fluorescence emission spectra of HSA + FA and pHSA have been observed which is an indication of the difference in the fluorophore micro-environment. The presence of fatty acid causes certain conformational changes which are monitored through the altered microenvironment around the fluorophore and thus there is a red-shift in the \( \lambda_{\text{max}} \) of emission in case of pHSA. However, the local conformational changes could not interfere with the active site geometry of the protein. Thus the function of the protein remains unchanged. Maybe the conditions for isolation, processing of pHSA and its storage could be responsible for the local conformational changes observed. On the other hand, examining the comparative intrinsic (Trp) fluorescence spectra of both rHSA and pHSA in presence of FA reveals that there is no significant difference in the \( \lambda_{\text{max}} \) of emission as well as the emission intensity keeping the activity level intact. This is indicative of the fact that the presence of fatty acid, in both the proteins, is not causing any significant changes to the tryptophan micro-environment. The type of local conformational changes sensed by the alteration of tyrosine microenvironment is insensitive to towards the tryptophan fluorescence. Hence it could be stated that the overall structural similarity exists between the two variants of the protein i.e. E. coli-derived rHSA and pHSA.

Interestingly it could be deciphered that the addition of FA helps stabilization of the structure of the E. coli-derived rHSA. It has been reported previously in numerous reports that the presence of FA helps in stabilization of the pHSA (Charbonneau et al., 2009; Curry et al., 1999; Dobretsov et al., 2012; Minchiotti et al., 1999). The same holds true for the rHSA as well in the present study. In the current case, sodium caprylate has been shown to enhance the stability of the HSA protein when incorporated in the protein fraction. The caprylate ion is known to prevent the aggregation of HSA and providing it the best conformation that stabilizes its structure (Cordes et al., 2012; Faroongsarng and Kongprasertkit, 2014). The E. coli-derived HSA fraction was hence stored in presence of FA. Whereas, the FA-free form of the HSA was always prepared by removal of FA from the rHSA + FA fraction to prevent its destabilization and aggregation in the solution form.

In numerous publications esterase activity of HSA has been exploited to study the quality of the protein and the functional status of protein has been described with the help of this activity assay (Ahmad et al., 2012; Anraku et al., 2001; Rabbani et al., 2017; Ranjar et al., 2013; Salvi et al., 1997). Denatured HSA does not exhibit esterase activity whereas only properly folded HSA exhibits the enzymatic activity (Dirks and Boyer, 1951; Means and Binder, 1975). This important functional aspect exhibited by the protein has been covered in this communication. In the present case, we have found that the E. coli-derived rHSA exhibited the esterase activity indistinguishable from the native form of HSA (Fig. 2b). One of the most important biological activities of HSA is its binding capacity for various ligands. We have exploited this property to explore the similarity among the two forms of HSA derived from different sources. The binding assay showed that the affinity of naproxen to E. coli-derived rHSA was similar to that of pHSA (Fig. 2c). Thus, indicating that no significant difference in drug-binding affinity exists between HSA and rHSA. The results obtained were also in agreement with the earlier published reports on pHSA (Cheruvallath et al., 1996; Lammers et al., 2013).

The structural stability aspects of the rHSA were also explored and compared with the plasma-derived form. Interestingly, the structural analysis revealed that the rHSA (in presence of FA) displayed similar thermal and equilibrium unfolding characteristics as compared to the pHSA. The calculated values of \( T_m \) values shed light on the fact that the presence of FA has helped the protein retain the native structure when subjected to a higher range of temperature. The present results are in agreement with the previously reported articles which had shown the FA mediated enhancement of the pHSA stability (Faroongsarng and Kongprasertkit, 2014). Furthermore, the data suggested that the folding pathway of both the proteins involved no intermediate formation as experimental data were best fitted to the two-state model. The thermal unfolding and refolding pattern adopted by the rHSA (with FA) is indistinguishable from that of the pHSA. The results are in concordance with the previously published reports on thermal unfolding and refolding of pHSA (Mitra et al., 2007). From the denaturant-mediated equilibrium unfolding data, it could be easily discerned that the HSA (with FA) appeared to be far stable than the HSA in absence of FA. The calculated \( D_m \) values for the GdnHCl-induced unfolding of HSA probed by CD and intrinsic fluorescence are around 2.3 M and 2.2 M respectively. Similar equilibrium unfolding pattern and \( D_m \) values around 2.0 M were obtained for GdnHCl mediated equilibrium unfolding of pHSA in the earlier reports (Santra et al., 2005).

In the present communication, we have been able to characterize the E. coli-derived rHSA and discern its similarity with the plasma-derived form in terms of its biological properties, functions, biochemical and biophysical properties. As per our knowledge, it is for the first time that the E. coli-derived rHSA, isolated majorly as a soluble fraction from the host, has been characterized and compared to its naturally occurring biological equivalent pHSA. Interestingly, as per the recent global albumin market survey of 2017–2025, it has been anticipated that the demand for HSA will increase tremendously in the market. Therefore an efficient and affordable alternate method to produce a substitute for HSA will be the prime requirement. Currently, in the market, yeast and transgenic rice (Oryza sativa) are the leading hosts exploited for the recombinant HSA production for its biotechnological applications, but they have limitations associated with them. The yeast system requires intense and complicated downstream processing protocols which make it impossible to produce rHSA in a cost-effective manner (Chen et al., 2013). Transgenic rice as a source requires acres of isolation zones for HSA preparation and there exists the risk of possible escape of transgenic rice gene into the environment from the field trail (He et al., 2011; Zhang et al., 2013). Transgene spread is a major environmental and food safety concern. E. coli, on the other hand, has always been the preferred microbial cell factory for recombinant protein production because of the mentioned advantages. Promoting a successful functional product preparation by using E. coli as a host is itself an advantage because of the simplicity and low-cost product preparation (Rosano and Ceccharelli, 2014; Tripathi et al., 2009). Thus, E. coli-derived rHSA seems to be a promising and competitive product against its biosimilars currently available in the market. In the present report, we have introduced E. coli-derived rHSA as an alternate source of HSA for its various biotechnological or non-clinical applications. Furthermore, in the present case, the protein is his-tagged and the purity levels are not suitable for its clinical usage yet, but the product is suitable for its biotechnological applications which hold a major share in the demand for the protein. We believe that the purification protocol described here could be further optimized for the large-scale operations. Moreover, efforts are currently going on in our lab to develop an E. coli-derived rHSA as a product for its clinical use as well. However, in this communication, we have proved that the rHSA produced by the E. coli reported here is similar to the native protein isolated from human blood plasma in terms of its biological, functional, biochemical and biological properties and can be promoted to be utilized to fulfill the demand for the biotechnological applications of the protein.
Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.03.004.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.03.004.

References


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None.