J Fluoresc (2014) 24:967–974
DOI 10.1007/s10895-014-1381-4

ORIGINAL PAPER

Bilirubin Binding with Liver Cystatin Induced Structural and Functional Changes

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Received: 16 January 2014 / Accepted: 19 March 2014 / Published online: 8 April 2014

Abstract Cysteine proteinases and their inhibitors play a significant role in the proteolytic environment of the cells. Inhibitors of cysteine proteinases regulate the activity of these enzymes helping in checking the degdration activity of cathepsins. The bilirubin secreated by liver cells can bind to cystatin present in the liver resulting in its functional inactivation, which may further lead to the increase in cathepsins level causing liver cirrhosis. In case of some pathophysiological conditions excess bilirubin gets accumulated e.g. in presence of *Fasciola hepatica* (liver fluke) in mammals and humans, leading to liver cirrhosis and possibly jaundice or normal blockade of bile duct causing increased level of bilirubin in blood. Protease-cystatin imbalance causes disease progression. In the present study, Bilirubin (BR) and liver cystatin interaction was studied to explore the cystatin inactivation and structural alteration. The binding interaction was studied by UV-absorption, FT-IR and fluorescence spectroscopy. The quenching of protein fluorescence confirmed the binding of BR with buffalo liver cystatin (BLC). Stern-Volmer analysis of BR-BLC system indicates the presence of static component in the quenching mechanism and the number of binding sites to be close to 1. The fluorescence data proved that the fluorescence quenching of liver cystatin by BR was the result of BR–cystatin complex formation. FTIR analysis of BR-Cystatin complex revealed change in the secondary structure due to perturbation in the microenvironment further confirmed by the decreased caseinolytic activity of BLC against papain. Fluorescence measurements also revealed quenching of fluorescence and shift in peak at different time intervals and at varying pH values. Photo-illumination of BR–cystatin complex causes change in the surrounding environment of liver cystatin as indicated by red-shift. The binding constant for BR-BLC complex was found to be 9.279×10^4 M^{-1}. The cystatin binding with bilirubin has a significant biophysical and pathophysiological significance, hence our effort to study the same.

Keywords Cystatin · Bilirubin (BR) · FTIR · Fluorescence

Introduction

Bilirubin, a metabolite of the heme in senescent red blood cells, is normally conjugated with albumin to form a water-soluble complex [1, 2]. Free bilirubin is toxic, and hence is transported to the liver as a complex with albumin where it is normally conjugated and excreted into the bile. Liver damage and related malfunctions can result in hyperbilirubinemia, i.e. an elevated level of unconjugated (or free) bilirubin in the blood. Excess free bilirubin tends to deposit in tissues, especially in the brain. Disorders in the metabolism of bilirubin, especially common among newborn infants, may cause jaundice, a yellow discoloration of the skin and other tissues [3, 4]. Accumulation of unconjugated bilirubin (UCB) in the central nervous system contributes to cell damage during severe neonatal hyperbilirubinemia and Crigler-Najjar type I syndrome leading to bilirubin encephalopathy [5, 6]. Bilirubin is a linear tetrapyrrole whose conformation is affected by internal hydrogen bonds formed between the carboxyl side chains and dipyrromethenone rings [7].

Bilirubin-IXa binds tightly to human serum albumin (HAS) at apparently only the high affinity site, forming a 1 :1 association complex [8, 9]. The proclivity of bilirubin to form association complexes with serum albumin and other proteins [8–11] is one of the properties that dominate the transport and metabolism of the pigment in vivo [8, 10, 12, 13].

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Cystatins are protein inhibitors of cysteine proteases of the papain family [14–16]. Cystatins inactivate target proteases reversibly and competitively by indirect blockage of the catalytic centers of these enzymes, thereby preventing substrate docking and cleavage. Cystatins are widely distributed in animals, plants and protozoa, both intracellularly and extracellularly, which indicates an important physiological role [14, 15, 17]. The members of the cystatin superfamily were grouped into three families on the basis of their location, size, and complexity of polypeptide chains [14, 18]. Members of family 1, the stefins, are found primarily intracellularly, contain about 100 amino acid residues (−11 kDa), and lack disulfide bonds. The human cystatins A and B, as well as rat cystatin α and β are representatives of this family. Members of family 2 cystatins are found primarily in body fluids but in tissues also. These contain about 120 amino acid residues (−14 kDa) and two intrachain disulfide bonds. The salivary cystatins, cystatin C, and chicken egg white cystatin belong to this family. Family 3, the kininogens, is found in the plasma and secretions of mammalian species. Three types of kininogens, the low-molecular-weight (LMWK), high-molecular-weight (HMWK), and T-kininogens are single-chain glycoproteins with molecular weight of −50 to 120 kDa. They are comprised of three Cystatin like domains that resulted from gene duplication. They contain additional disulfide bonds and are also glycosylated. Kininogens have an additional polypeptide at the C terminus containing the bradykinin sequence, which can be cleaved by kallikrein [14, 15, 18].

Materials

Papain, CNBr activated sepharose-4B, casein, acrylamide, ethylene diamine tetra acetic acid (EDTA), acetone, Coomassie Brilliant Blue R-250 and cysteine were from Sigma Chemical Company, St. Louis, USA. Medium-range molecular weight markers were from Genei. BR was purchased from Sisco Laboratories, India. All other chemicals used were of analytical grade.

Methods

Purification of Liver Cystatin

Cystatin was isolated and purified from buffalo liver by using a three-step procedure. Fresh liver tissue (100 g) was homogenized in 200 ml extraction buffer (0.05 M sodium phosphate buffer, pH 7.5, 1.0 % NaCl, 3 mM EDTA, 2.0 % n-butanol). The homogenized tissue was subjected to alkaline treatment. The supernatant of pH treatment was then subjected to 40 % ammonium sulphate saturation. The supernatant obtained was thereafter put to 40 to 60 % ammonium sulphate saturation. The precipitated protein after extensive dialysis was applied to a Cm-papain-Sepharose, where affinity of desired thiol protease inhibitor for papain lead to its purification. Eluted fractions were analysed for protein concentration and inhibitory activity. Purified liver cystatin (BLC) gave a single band on polyacrylamide gel electrophoresis (PAGE). The molecular weight of the inhibitor was found to be 17.5 kDa as calculated by its subunit structure on sodium dodecyl sulphate (SDS) PAGE.

Protein Estimation

Protein content was quantitated by Folin's phenol reagent by the method of Lowry et al. (1951) [19].

Assay of Thiol Proteinase Inhibitory Activity

The inhibitory activity of cystatins was assessed by its ability to inhibit caseinolytic activity of papain by the method of Kunitz (1947) [20].

Preparation of Solutions

Stock solution of bilirubin was prepared by dissolving 2 mg bilirubin in 2 ml 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M sodium carbonate and 1 mM EDTA and immediately diluting it to the desired volume with the above buffer. The solution was filtered and stored in the dark. The concentration of bilirubin was determined spectrophotometrically by taking the absorbance of the bilirubin solution at 440 nm using a molar extinction coefficient of 47,500 M^{-1} cm^{-1} [21]. The solution was prepared fresh and used within 2 h. All experiments involving bilirubin were done in dark.

Absorption Spectroscopy

Absorption spectra of cystatin as well as cystatin bound to BR were taken on UV–vis spectrophotometer in the wavelength range 220–400 nm using a cell holder with 1 cm path length.

Fluorescence Spectroscopy

Fluorescence measurements were made on a Shimadzu spectrofluorometer (25 °C) using a quartz cell with 1 cm path length. The excitation and emission slits were set at 5 and 10 nm, respectively. The fluorescence of BR bound to cystatin was recorded in the wavelength range 250–400 nm after exciting the complex at 280 nm. Photochemical experiments were carried out to measure photo-induced changes in the optical properties of the BR–cystatin complex. Samples were exposed to 40-W white fluorescent light (FTL; Anchor Electronics and Electricals Ltd., India) for various time periods. The distance of the sample from the light source was

2 cm. Photo-induced changes in the BR–cystatin complex were measured by fluorescence and absorption measurements.

Fourier Transform Infrared Spectroscopy

Infrared spectroscopy was done to see the conformational changes observed during cystatin and bilirubin interaction at relevant concentrations. The spectra was truncated between 1900 and 1220 cm^{-1} and baseline corrected. The equipment used was NICOLET (ESP) 560 spectrophotometer equipped with a transmission OMNIC ESP 5.1 software and a DTGS detector; data was analyzed and quantitated using Grams 32 software. Original spectra of native cystatins along with cystatin co-incubated with Bilirubin at 37 °C were taken with a fixed concentration of cystatin (2 μM) and an increasing concentration of Bilirubin (1–9 μM) with a resolution of 4 cm^{-1} and 128 scans. The changes in peak frequency and intensity were then assigned to conformational changes within the protein [22].

Statistical Analysis

All experiments were repeated three times to document reproducibility. Wherever applicable, data are expressed as mean ± standard error of the mean (SEM). Significance of difference in mean values was evaluated using one-way analysis of variance (ANOVA). A probability level of P\0.05 was selected as indicating statistical significance.

Results and Discussion

BR Binding Studies

Binding of BR to liver cystatin (LC) was studied using fluorescence, absorption spectroscopy and FTIR. To a fixed concentration of protein (1 μM) taken in various test tubes, increasing concentrations of BR were added. Absorption and fluorescence spectra were recorded after incubating the solution for 30 min. Absorption spectra were recorded in the wavelength range 220–400 nm. Fluorescence was recorded in the wavelength range 250–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. All spectral measurements were done in dim light to prevent undesired photo-degradation of BR. Binding of BR to cystatin resulted in a blue-shift from 340 to 335 nm. Increasing the concentration of bilirubin resulted in increased

BLC (2 μM) was incubated with various concentrations of Bilirubin from 0.25 to 20 μM for 30 mins. The fluorescence was recorded in wavelength region 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence. The slits were set at 5 and 10 nm for excitation and emission. The path length of the sample was 1 cm.

Quenching of the cystatin–BR complex. Figure 1 shows the fluorescence emission spectra of the complex of BR with cystatin in the presence of increasing concentration of BR. It was found that maximum quenching occurred at 20 μM bilirubin concentration.

The fluorescence data were analysed by fitting to the Stern–Volmer equation: $F_0/F = 1 + K_{SV}[Q]$, where F_0 and F are the steady-state fluorescence in the absence and presence of quencher (bilirubin), respectively, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of the quencher (BR).

The plot of $Log(F_0-F)/F$ versus Log[Q] can be used to determine the binding constant and the number of binding sites, using the following equation given by Feng et al. (1998) and Gao et al. (2004) [23, 24]:

$$\mathrm{Log}[(F_0-F)/F] = \mathrm{Log}\ K + n\ \mathrm{Log}[Q],$$

where K and n are the binding constant and the number of binding sites, respectively (Fig. 2).

The binding constant was found to be $9.279\times10^4\ M^{-1}$ and the number of binding sites was found to be 1. The absorption spectra of cystatin with BR were also computed and in accordance with the fluorescence results, binding of BR to inhibitor was evidenced by increase in absorbance and red-shift. The results are in accordance with the binding of BR with albumin [4, 25–27].

The fluorescence quenching data was further analyzed by stern-volmer equation as described in "Methods". The plot of F_0/F vs log [Q] of bilirubin gives binding constant (K) and the number of binding sites (n) between BR-BLC complex. Regression R^2=0.914.

BLC was incubated with different concentrations of BR (0.25–1 μM) for 30 mins. The absorbance spectra were taken in the region 1800–1400 cm^{-1} as described in "Methods".

Functional Inactivation of Liver Cystatin

Effects of bilirubin on liver cystatin function were assessed by monitoring the changes in its anti-proteolytic activity using caseinolytic assay of papain (Kunitz 1947) [19]. Liver cystatin (1 μM) was incubated with increasing concentrations of

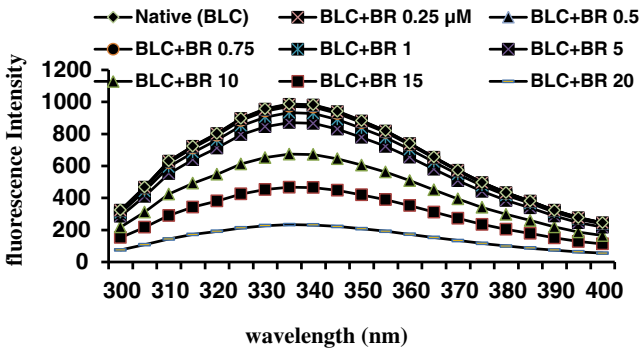

Fig. 1 Intrinsic fluorescence study of BLC in the presence of BR

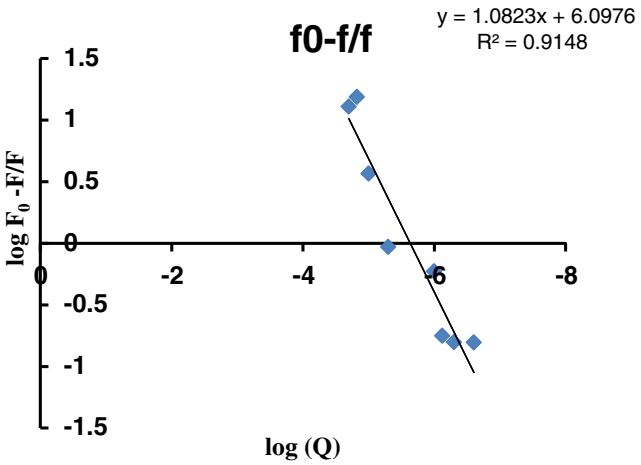

Fig. 2 Stern-Volmer plot for the binding of BLC with BR at different concentrations

bilirubin (1–10 μM) for 30 min. The results obtained are summarized in Table 1. As shown in the Table 1, exposure of liver cystatin to bilirubin (1 μM) resulted in rapid decline of anti-proteolytic (39 %) loss towards papain, with half of the inactivation (56 %) taking place at 3 μM bilirubin concentration. More than 80 % loss in activity took place upon incubating the cystatin with 10 μM BR.

Effect of pH on Cystatin-BR Complex

The effect of pH on fluorescence of cystatin–bilirubin complex is shown in Fig. 3. The cystatin–BR complex shows a two-step transition in the pH range 6–9. In the pH range 6–7 there is blue-shift of 5 nm from 340 to 335 nm along with a decrease in fluorescence intensity. However, in the pH range 8–9 there is a red-shift of 5 nm from 340 to 345 nm, accompanied by a significant increase in fluorescence intensity. Changes in the state of ionization in the pH range 6–9 of various ionizable groups of bilirubin (pyrrole nitrogens, propionic acid, carboxyl groups etc.) may change the mode of interaction between the complex components. The difference absorption spectrum (the system containing bilirubin and cystatin measured against cystatin alone) in the ultraviolet region in the pH range 6–9 is shown in Fig. 3 (inset). The difference spectra shows a peak at 250 nm, suggesting the formation of the cystatin–bilirubin complex, with no indication of dissociation of bilirubin. The gradual increase in absorption in the pH range 6–9 is most likely due to conformational changes of the bilirubin molecule bound at specific sites. There was a loss in inhibitory activity of cystatin upon decreasing the pH to 6 or increasing the pH to slightly alkaline conditions, binding of BR with albumin [4, 25–27].

BLC (2 μM) was incubated with various at different pH buffers (7–11) in presence of BR 0.5 μM for 30 mins. The fluorescence was recorded in wavelength region 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence.

With BLC concentration fixed at 2 μM while the BR concentration was taken as 0.5 μM in solution varying in pH values from 7 to 10 at room temperature in sodium phosphate buffer.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was done to analyze the conformational changes during cystatin-BR interaction. The results further confirm the damage caused to cystatin as shown in previous investigations. In the IR spectra of proteins, the secondary structure is most clearly reflected by the amide I and II bands, particularly the former [28–30]; the amide I band absorbs at 1657 cm^{-1} (mainly a C = O stretch), and the amide II band absorbs at 1542 cm^{-1} (C-N stretching coupled with N-H bending modes) [31, 32]. It has also been reported that, for a native protein, the amide I component for the α-helical structure locates at 1656 (2 cm^{-1} and the band components for the α-sheet structure should locate between 1622 and 1642 cm^{-1}(lower wavenumber α-sheet bands) and between 1690 and 1698 cm^{-1} (higher wavenumber β-sheet bands) [23–25, 28]. Figure 4 shows the original spectra of native (untreated cystatin) along with cystatin co-incubated for 30 mins consecutively with bilirubin (0.25–1.0 μM). Upon bilirubin interaction, the α-helix structure was reduced with increase in the bilirubin concentration in favour of β-sheet structure for liver cystatin. The reduction of the α-helix in favour of the β-sheet structure is indicative of the partial unfolding of the inhibitor in the presence of bilirubin. A similar conformational transition from α-helix to β-sheet structure was observed for the myelin protein upon heating the protein at 35 °C [29].

Table 1 Inhibitory activity of BLC in the presence of bilirubin at different concentrations

Concentration of bilirubin (BR) (μM)	0.25	0.5	0.75	1.0	5.0	10	15	20
% Remaining activity	85±3.2*	62±1.3*	55±2.5*	42±1.1*	29±2.2*	14±3.1*	11±3.3*	6±4.2*

Liver cystatin (BLC) (1 lM) was incubated at different concentrations of bilirubin for 30 min

BLC was assayed for loss in anti-proteolytic activity by caseinolytic method of Kunitz (1947). Activity of native LC is taken to be 100

Results are mean ± SEM of three or more separate experiments

* Significantly different from native LC (control) at *P*\0.05 by one-way ANOVA

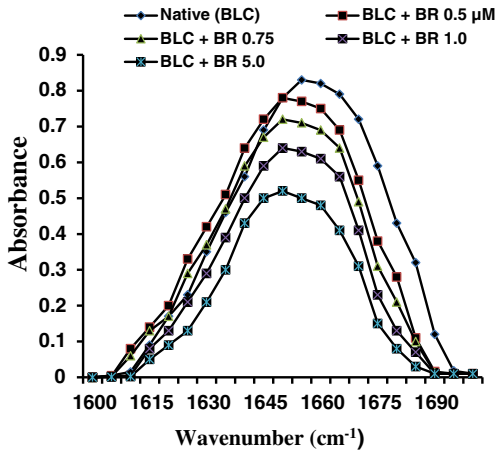

Fig. 3 FT-IR spectra of BLC in the presence of BR

Thus, the results summarized in of Fig. 4 further confirm the change in the secondary structure and corresponding functional inactivation caused to cystatin as evidenced by a significant shift in the peak intensity, i.e., from 1656.08 to 1621.55 and significant changes in the structure of cystatin from that of α-helix to β structure (lower wavenumber β-sheet bands), after co-incubating cystatin (1 and 2) for 4 h with bilirubin (3 mM).

Native protein (BLC) 2 μM was treated with BR and fluorescence spectra of BR cystatin complex obtained for different time of exposure to white light. Protein (BLC) native, unexposed BR-BLC complex, exposed for 5, 10, 20, and 30 min. The fluorescence was recorded in wavelength region 300–400 nm after exciting the protein solution at 280 nm for total protein fluorescence.

Native protein (BLC) 2 μM and BR (0.5 μM) complex was exposed to white light and scans obtained for different time of exposure to white light. The unexposed LC-BR complex was exposed for 5, 10, 20, and 30 min.

Effect of Photo-Illumination on BR–Cystatin Complex

Effect of photo-illumination of BR–cystatin complex was studied by fluorescence spectroscopy. As can be seen in Fig. 4, there was a red-shift of 5 nm accompanied by the decrease in the fluorescence intensity, the maximum decrease being for 30 min exposure. The conformational twisting of bound BR leading to configurational or structural isomerisation upon photo illumination was responsible for photo-induced fluorescence changes. The difference absorption spectra for BR cystatin complex under white light for varying time periods (0–30 min) are shown in Fig. 4. A blue-shift of 5 nm was observed upon 10 min photo-illumination. A significant blue-shift of 10 nm was observed upon increasing the time of exposure to 30 min. The significant blue-shift indicates the sensitivity of the BR–cystatin complex to white light. Photo-illuminated BR–cystatin complex lost its papain

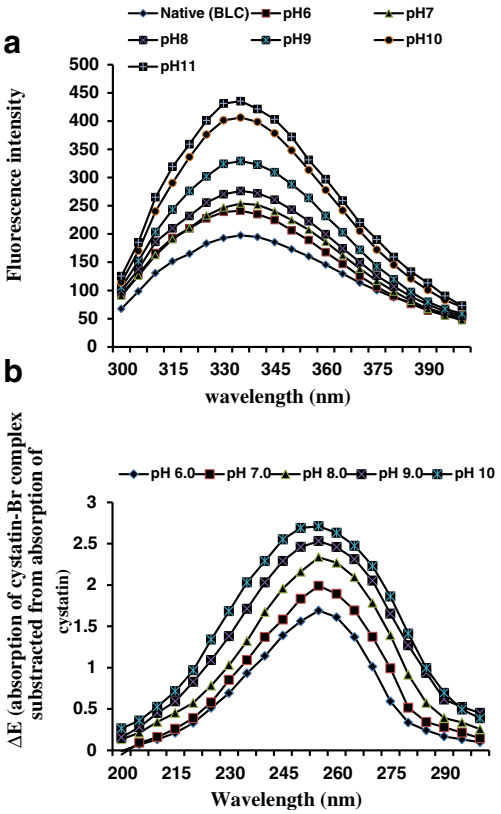

Fig. 4 **a** Intrinsic fluorescence study of BLC in the presence of BR at different pH values. **b** UV–vis spectroscopy of BLC in the presence of BR at different pH values

inhibitory activity, suggesting that functional inactivation of protein occurs upon exposing the complex to white light.

Discussion

Bilirubin (BR) remains a biologically important, chemically interesting and pedagogically useful molecule. BR is a bile pigment that is produced from the catabolism of haemoproteins at a rate of about 0.25–0.5 g per day and has toxic effects on many cellular functions [33–35].

Fluorescence spectroscopy is a useful technique which provides useful information on the interactions of macromolecules with proteins. Intrinsic fluorescence of proteins provides a considerable information about the protein structure and dynamics and has been used extensively to study protein folding and association reactions. Various measurable parameters of fluorescence viz. Quenching, enhancement of intensity, spectral shift etc. are used for interpretation of related structure-dynamics in proteins [36]. When different concentrations of bilirubin (BR) solutions were incubated with a fixed concentration of cystatin, a remarkable decrease in fluorescence intensity of cystatin was observed, which indicated that BR could bind with cystatin (Fig. 1). Fluorescence quenching is the decrease of the quantum yield of fluorescence from a

fluorophore induced by a variety of molecular interactions with a quencher molecule [37]. Talking advantage of our finding that bilirubin can strongly quench the fluorescence intensity of cystatin, stern-volmer plot further confirmed that the binding between bilirubin and cystatin is purely static. Stern-volmer analysis of fluorescence quenching data showed the binding constant to be 9.279×10^4 M^{-1} and the number of binding sites equal to one (Fig. 2). Binding constant determines the affinity of the ligand (bilirubin) with protein (cystatin). The value of the binding constant is in accordance with that reported for the binding of methotrexate with goat lung cystatin [38] and interaction of bilirubin with liver cystatin [39].

The absorption spectra of cystatin with BR were also computed and in accordance with the fluorescence results binding to BR inhibitor was evidenced by increase in absorbance and red shift thus confirming the binding of cystatin to BR. The results are in accordance with the binding of BR with albumin [4, 25–27].

Upon complexation with bilirubin the thiol proteinase inhibitory activity was severely challenged suggesting that the binding of BR to cystatin was accompanied by functional inactivation of cystatin (Table 1). A similar decline in papain inhibitory activity of cystatin was seen upon binding of methotrexate with lung cystatin suggesting changes in the environment of the crucial amino-acid residues of the protein [38] and interaction of bilirubin with liver cystatin [39].

FTIR spectroscopy was done to analyze the conformational changes during cystatin-Bilirubin interaction. The results confirm the damage caused to cystatin as shown in previous investigations. In the IR spectra of proteins, the secondary structure is determined by the amide I and II bands, particularly the former [28–30]; the amide I band absorbs at 1657 cm^{-1} (mainly a C = O stretch), and the amide II band absorbs at 1542 cm^{-1} (C-N stretching coupled with N-H bending modes) [31, 32]. Figure 3 shows the original spectra of native (untreated cystatin) along with cystatin co-incubated for 30 min with bilirubin (0.5–5 μM). It was observed that upon bilirubin interaction, the α-helix structure was reduced as is evidenced by the decrease in intensity around amide I (1655 cm^{-1}) region in the liver cystatin (BLC). There is also a shift in peak in amide I region from 1655 towards 1637 cm^{-1} with increase in the bilirubin concentration. The reduction of the α-helix in favor of the unordered structure towards the β-sheet conformation is indicative of the partial unfolding of the inhibitor in the presence of bilirubin. A similar conformational transition from α-helix to β-sheet structure was observed for the myelin protein upon heating the protein at 35 °C [29].

The results summarized in parts Fig. 3 further confirm the damage caused to cystatin as evidenced by a significant shift in the peak intensity, i.e., from 1655 to 1637 and significant changes in the structure of cystatin from that of α-helix towards β structure after co-incubating cystatin (BLC) for 30' with bilirubin (0.5–5 μM).

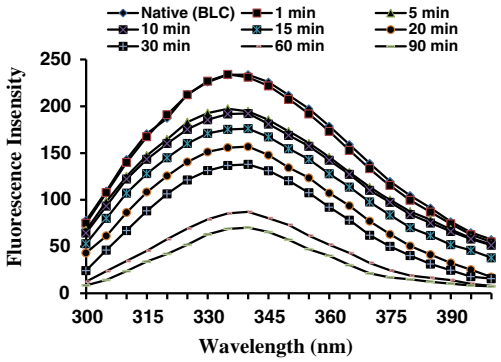

Fig. 5 Intrinsic fluorescence study of BLC in the presence of BR at different time intervals

We extended the studies to cover the effect of pH, time and photo irradiation on the cystatin-bilirubin complex. The cystatin-BR complex used for further study was in the ratio of (1:1). The cystatin-bilirubin complex was incubated in the buffers of different pH (6.0, 7.0, 8.0, 9.0 and 10.0) and same ionic strength. In the pH range of 6.0 to 7.0 fluorescence intensity was found to increase accompanied with a blue shift. However, in the pH range of 7.0 and 9.0, a red shift was observed accompanied by a significant increase in fluorescence intensity (Fig. 4a). Further there was an increase in fluorescence intensity in the pH range of 9.0 to 11.0 with a slight blue shift. Changes in the state of ionization in the pH range between 6.0 and 9.0 of various ionisable groups of

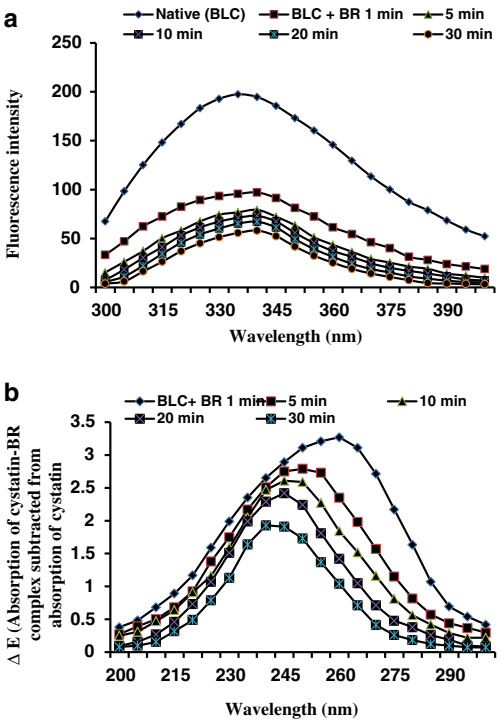

Fig. 6 **a** Intrinsic fluorescence study of BLC-BR in presence of Fluorescent light. **b** UV–vis spectroscopy of BLC-BR complex in presence of Fluorescent light

bilirubin (pyrrole nitrogen, propionic acid and carboxyl groups) may change the mode of interaction between the complex components. In case of pH 10 and pH 11 the slight blue shift indicates the refolding or transition in the structure towards a more compact form. It can be due to extreme change in the pH towards the alkaline side but there was no regaining of inhibitory activity at higher pH (pH 10–pH 11). These observations were further confirmed with supporting results from UV–vis spectrophotometer. The absorption spectra of cystatin with BR shows a peak at 225 nm suggesting the formation of cystatin bilirubin complex with no indication of dissociation of bilirubin (Fig. 4b). The gradual increase in absorption in the pH range of 6.0 to 10.0 is most likely due to conformational changes of the bilirubin molecule bound at specific sites. There was a loss in inhibitory activity of cystatin-BR complex upon decreasing the pH to 6.0 or increasing the pH to slight alkaline conditions, thereby suggesting that conformational changes are accompanied by functional inactivation of cystatin. Similar conformational transition have been reported upon varying the pH of the BSA-bilirubin complex [40].

To investigate the effect of varying time of incubation of cystatin with bilirubin on the activity of cystatin, the complex was incubated for varying time intervals 15'–120'. Results indicated that increasing the time of incubation led to a sharp decrease in fluorescence intensity, which tended towards a plateau when the fluorescence intensity had reached about three-fourth of its initial value (Fig. 5).

Irradiation of BR-cystatin complexes under white light for varying time periods (0–30') was also studied by fluorescence and UV spectroscopy. The decrease in the fluorescence intensity along with a red shift of 5 nm was seen upon exposing the BR-cystatin complex to white light (Fig. 6a). These photo-induced changes could be due to structural-isomerazation resulting in the conformational twisting of the bound BR. The photochemical properties of different bilirubin-albumin complexes of human and other mammalian sources have been reported by Khan et al. (2002) [41]. The photo-induced changes were also confirmed by UV spectroscopy. Upon exposing the BR-cystatin complex towards white light (Fig. 6b). These results are in accordance with that of lamola (1983) [42]. Photo-illuminated BR-cystatin complex lost its papain inhibitory activity as compared to the unexpected BLC-BR complex (1:1) used for the study suggesting that the functional inactivation of complex occurs upon exposing the complex to white light.

Conclusions

There is a plausible correlation between bilirubin and cystatin which may play a significant role in various hepatic diseases such as jaundice characterized by high plasma bilirubin levels [43]. The accumulated bilirubin may bind to liver cystatin resulting in its functional inactivation, which may further lead to the increase in cathepsins level, the hallmark of liver cirrhosis. Such correlations may have significant advantages in clinical interpretations and warrant further study to elucidate the underlying mechanisms. Further there is a need to determine the effective and safe ways to clear the body of the excess bilirubin in case of some pathophysiological conditions like presence of *Fasciola hepatica* (liver fluke) in case of mammals and humans, which act as host to this parasite leading to liver cirrhosis and possibly jaundice or normal blockade of bile duct causing increased level of bilirubin in blood.

Our study reveals correlations between bilirubin and cystatin which may play a significant role in various hepatic diseases such as jaundice, which is accompanied by increase of bilirubin levels. The accumulated bilirubin upon binding with liver cystatin results in its functional inactivation, which further leads to increase in cathepsin levels, the hallmark of liver cirrhosis. Such correlations may have significant advantages in clinical interpretation and warrant further study to elucidate the underlying mechanisms.

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