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ARTICLE

Interaction of Curcumin with β-Lactoglobulin—Stability, Spectroscopic Analysis, and Molecular Modeling of the Complex

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Curcumin (diferuloyl methane) is the physiologically and pharmacologically active component of turmeric (*Curcuma longa* L.). Solubility and stability of curcumin are the limiting factors for realizing its therapeutic potential. β-Lactoglobulin (βLG), the major whey protein, can solubilize and bind many small hydrophobic molecules. The stability of curcumin bound to βLG in solution is enhanced 6.7 times, in comparison to curcumin alone (in aqueous solution). The complex formation of curcumin with βLG has been investigated employing spectroscopic techniques. βLG interacts with curcumin at pH 7.0 with an association constant of $1.04 \pm 0.1 \times 10^5$ M^{-1} to form a 1:1 complex at 25 °C. Entropy and free energy changes for the interaction derived from the van't Hoff plot are 18.7 cal mol^{-1} K^{-1} and −6.8 kcal mol^{-1} at 25 °C, respectively; the interaction is hydrophobic in nature. The interaction of βLG with curcumin does not affect either the conformation or the state of association of βLG. Competitive ligand binding measurements, binding studies with denatured βLG, effect of pH on the curcumin−βLG interaction, Förster energy transfer measurements, and molecular docking studies suggest that curcumin binds to the central calyx of βLG. These binding studies have prompted the preparation and encapsulation of curcumin in βLG nanoparticles. Nanoparticles of βLG prepared by desolvation are found to encapsulate curcumin with >96% efficiency. The solubility of curcumin in βLG nanoparticle is significantly enhanced to ∼625 μM in comparison with its aqueous solubility (30 nM). Nanoparticles of βLG, by virtue of their ability to enhance solubility and stability of curcumin, may fit the choice as a carrier molecule.

KEYWORDS: Curcumin; β-lactoglobulin; nanoparticle; fluorescence; energy transfer; binding constant

INTRODUCTION

Curcumin (diferuloyl methane), a yellow lipid-soluble phenolic dietary spice, present in the rhizome of turmeric (*Curcuma longa* L.), finds wide use as a coloring agent in food. Because of its potential health benefits, researchers have evinced great interest in this molecule. A wide range of pharmacological attributes of curcumin such as antioxidative, anti-inflammatory, antiangiogenic, anti-amyloid, anticancer, antimicrobial, wound-healing, and hepatoprotective properties have been well revealed (*1*). Biological activities of curcumin depend on its bioavailability and metabolism. Poor solubility, stability, and bioavailability in aqueous media limit the use of curcumin as an efficient medicine (*1*). Efforts are on to enhance the solubility of curcumin by making complexes with natural biodegradable carriers such as casein micelles, phospholipid complex, and serum albumin (*2*−*4*). Biodegradable polymers with encapsulated curcumin are proposed to be used as a vehicle for drug delivery to cancer cells or to treat inflammation (*2*, *4*).

Bovine β-lactoglobulin (βLG), a low molecular weight whey protein, is found in cow's milk (3 g/L). It is the most abundant protein in whey, constituting 40% of the protein component. βLG is a small globular extracellular protein—belonging to the lipocalins superfamily—capable of binding and transporting small hydrophobic molecules within its central cavity known as the calyx (*5*). Bovine βLG, which occurs as a dimer at neutral pH, manifests as a monomer at pH ≤ 2, with a molecular mass of ∼18000 Da (*6*). βLG, containing 162 amino acids, consists of nine β strands—A to I. The strands A−H form an up and down β-barrel with one major α-helix at the C-terminal end of the molecule (*6*). The molecule has two ligand-binding sites—a central calyx domain of the β-barrel and a surface hydrophobic pocket in the groove between the α-helix and the β-barrel (*7*).

The proposal of βLG as an oral drug carrier is supported by its ability to bind many biochemically important hydrophobic compounds (*5*). βLG is edible and easily solubilized with good emulsifying characteristics. Its cost effectiveness, abundant availability, and acceptability make it an attractive alternative protein that can be used as a carrier molecule for curcumin.

Encapsulation of bioactive molecules in nanoparticles improves its distribution and solubility (*8*). Food-based nanocomplexes not only provide protection against degradation by environmental stress (e.g., light, heat, oxygen, or pH sensitivity)

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but also improve bioavailability as well as stability of small molecules (*8*). Protein nanoparticles being biodegradable claim preference over other carriers due to their ability to incorporate a wide variety of drugs in a nonspecific fashion (*9*, *10*). Structural and physicochemical properties of milk proteins favor their choice as vehicles for encapsulation and controlled release of bioactive compounds (*11*, *12*).

The potential of βLG to be a carrier molecule for curcumin was investigated by following the interaction between curcumin and βLG using spectroscopic techniques. The binding site was visualized by molecular modeling. In an approach to enhance the solubility of curcumin, its encapsulation was evaluated using biocompatible and easily available whey protein βLG. Our study has suggested that curcumin can be complexed with a βLG molecule, which may serve as a useful carrier for curcumin.

MATERIALS AND METHODS

Curcumin, βLG (from bovine milk, ~90%), palmitate, and Trizma base were from M/S Sigma Aldrich Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade. All of the solutions were freshly prepared.

The molar concentration of curcumin, dissolved in methanol, was determined by measuring the absorbance at 425 nm, using the extinction coefficient $\varepsilon_{425\,nm} = 54954\ cm^{-1}\ M^{-1}$. Palmitate stock solution, prepared in absolute ethanol and purged with nitrogen gas, was kept in the darkness at −20 °C. The concentration of βLG, dissolved in 50 mM Tris HCl buffer, pH 7.0, was determined by absorbance measurement (at 278 nm) using either $\varepsilon_{278\,nm} = 17600\ cm^{-1}\ M^{-1}$ or $E_{1\,mg/mL}{}^{1\%} = 0.93$. The protein solution was centrifuged, before use, to remove any aggregates. Absorbance measurements were made on Shimadzu 1601 double beam spectrophotometer, using a 10 mm path length quartz cell.

Stability Measurements. To study the stability of curcumin at pH 7.0 with time, curcumin in buffer (Tris HCl, 50 mM) or in 1 mg/mL βLG was incubated at 30 °C. The concentration of curcumin at different time intervals was calculated by absorbance measurement at 425 nm. The half-life was calculated by fitting the data to a first order reaction.

Fluorescence Measurements. Fluorescence measurements were made on a Shimadzu RF 5000 spectrofluorimeter, attached with a thermostatic circulating water bath maintained at 25 °C. A 10 mm path length cell was used. The solution in the cuvette was stirred continuously with the help of Hellma cuv-o-stir. Excitation and emission slit widths used were 5 and 10 nm. Steady state fluorescence of curcumin was measured by fixing the curcumin concentration at 10 μM and adding aliquots of βLG from the stock solution, in the concentration range 0−38 μM. The emission spectra were recorded from 450 to 600 nm, with the excitation wavelength fixed to 430 nm. The fluorescence intensities of the sample were corrected from the fluorescence intensity of the curcumin solution without βLG. Similar experiments were carried out using denatured βLG. βLG was denatured by incubation at 80 °C in a preheated water bath for 10 min and then cooled immediately. Binding studies of curcumin with denatured βLG were carried out at 25 °C. An increase in the fluorescence intensity of curcumin at 505 nm was recorded. The data recorded at maximum emission wavelength were used to analyze the binding parameters from the following equation (*13*)

$$1/\Delta FI = 1/\Delta FI_{max} + 1/K_b \Delta FI_{max}[\beta LG] \quad (1)$$

where ΔFI is the change in the curcumin fluorescence in the presence of βLG, ΔFI_{max} is the maximal change in fluorescence intensity, K_b is the binding constant, and [βLG] is the concentration of protein added. The binding constant was calculated to examine the effect of pH on the binding of curcumin to βLG at different pH values (5.5, 6.5, and 7.0).

Intrinsic fluorescence of βLG was measured at 335 nm. The concentration of βLG was fixed at 2.95 μM; the spectra for each addition of curcumin were recorded from wavelength range 305−570 nm, when excited at 295 nm. At this low concentration of protein, βLG existed predominantly as a monomer (*14*). The fluorescence intensities of the sample were corrected for an inner filter effect by the addition of ligand to *N*-acetyl tryptophanamide solution. Quenching, as a function of curcumin concentration, was analyzed in terms of binding of curcumin to βLG using established procedures (*15*). The driving force for the binding of curcumin to βLG was analyzed by following the effect of change in temperature in the range 15−45 °C, on the binding constant of curcumin with βLG.

Fluorescence Resonance Energy Transfer (FRET). Efficiency of energy transfer, critical energy transfer distance (R_o), and the distance (r_o) between acceptor and donor were calculated according to Förster theory of energy transfer (*15*, *16*). The energy transfer efficiency *E* was computed from the equation

$$E = 1 - F/F_o \quad (2)$$

where F_o = fluorescence intensity of βLG alone and F = fluorescence intensity of βLG with ligand. The percentage energy transfer was also calculated using eq 2, by adding aliquots of βLG in the range 0−0.5 mg/mL to a fixed concentration of curcumin. The *Y*-intercept on the plot of βLG (mg/mL) against percentage energy transfer gave the total percentage energy transfer.

Circular Dichroism (CD). CD spectra in the ranges of wavelength 195−260, 260−320, and 320−500 nm were recorded on a Jasco J-810 spectropolarimeter. While measurements in far UV were carried out using 1 mm path length quartz cell, 10 mm path length quartz cells were used for near UV and the visible range. An average of three scans at a speed of 10 nm/min with a bandwidth of 1 nm and a response time of 1 s were recorded. The concentration of βLG used was 0.2 mg/mL in the far UV and 1 mg/mL in the near UV and visible range. The induced CD spectra were obtained by subtracting the CD spectra of the ligand−protein mixture from the spectra of protein alone. A mean residue weight of 115 was used to calculate the molar ellipticity value.

Effect of Curcumin on the Heat Denaturation of βLG. βLG (1 mg/mL) was dissolved in 20 mM phosphate buffer, pH 6.6. The solution was cooled immediately following heating to 85 °C for 10 min. For the reaction containing curcumin, an equimolar concentration of curcumin was added to the βLG solution. The unbound curcumin was removed by centrifuging the sample. The solution was then heated to 85 °C for 10 min and cooled immediately. Gel filtration was performed using a TSK-Super SW2000 (4.6 mm × 300 mm, 4.0 μm) column on a Waters high-performance liquid chromatography (HPLC) system (Waters, Milford, MA), equipped with a 1525 binary pump and Waters 2996 photodiode array detector. The column was equilibrated with phosphate buffer before injecting 20 μL of the above sample. The sample was eluted isocratically in the same buffer. The flow rate was maintained at 0.2 mL/min at 25 °C. Detection was at 280 nm.

Docking Studies. On the basis of experimental results, docking experiments were carried out to visualize the binding site of curcumin to βLG. To generate the binary complex of curcumin−βLG, the crystal structure of the complex of βLG−palmitate available at 2.5 Å resolution (labeled 1B0O in Brookhaven Protein Data Bank) was chosen as a template (*17*). The binding site and the mode of binding were identified by an automated public domain software package ArgusLab 4.0.1 (Mark Thompson and Planaria Software LLC) that performs the molecular constructions, calculations, and visualizations. The possible binding conformations and orientations were analyzed by clustering methods. Flexible docking was carried out using the genetic algorithm implemented by the program ArgusLab (AScore scoring method). This dock engine was set to perform an exhaustive search for automated docking with complete ligand flexibility to elucidate the mode of interactions between curcumin and βLG. The water molecules and palmitate were removed, and the hydrogens were added using the builder module of ArgusLab. The ligand structure was constructed in and submitted to the PRODRG site to get an optimized structure (*18*). The docking search was done for the whole protein without defining the target area or protein pocket. Geometry optimization was carried out with grid resolution of 0.4 Å and grid spacing of 35.76 Å × 47.68 Å × 41.95 Å. The pose with the minimal energy was taken as the optimal binding mode. To evaluate the effectiveness of the ArgusLab dock engine, redocking of palmitate back to βLG was done, and the resultant best pose was compared with the corresponding crystal structure of palmitate−βLG complex (PDB: 1B0O). The best returned pose revealed similar interactions. This validated the docking accuracy needed for further studies with curcumin.

Preparation of Encapsulated Curcumin and Characterization. The nanoparticle of βLG was prepared according to the method of Gunasekaran et al. (*12*). To the βLG particles, dispersed in water containing

0.002% sodium azide, a known amount of curcumin was added. The solution was vortexed, along with sonication before being kept for equilibration. The unbound curcumin was removed by centrifuging the sample at 12000*g* for 20 min, which pulls down only the undissolved curcumin. Encapsulated sample or the control was characterized using a scanning electron microscope (SEM) (Leo 435 VP, Cambridge, United Kingdom).

For SEM analysis, a drop of the sample was placed on a microscopic coverslip, vacuum-dried, and coated with gold before being observed under microscope. The morphology of the nanoparticle was evaluated. The average size and distribution of the nanoparticle were determined using Zetasizer Nanoseries (Nano ZS, Malvern Instruments Ltd., Malvern, United Kingdom). The measurements were carried out at 25 °C. The dispersion pH was 7.0 for both the control and the sample containing the curcumin. An average of 10 measurements was used to report the size.

Encapsulation Efficiency and In Vitro Release of Curcumin Bound to *β*LG Nanoparticle. Encapsulation efficiency of curcumin bound to βLG was determined by adding curcumin (0.25 mg) in 10 mg/mL solution of βLG nanoparticle. The solution was equilibrated for 30 min in an incubator equipped with a shaker and was maintained at 25 °C for 30 min. The solution was centrifuged for 20 min at 12000*g* to pellet the undissolved curcumin. The pellet was carefully dissolved in a known amount of methanol, and curcumin was quantified, spectrophotometrically, at 425 nm.

$$\text{encapsulation efficiency (\%)} = \frac{(\text{total amount of curcumin} - \text{free curcumin})}{\text{total amount of curcumin}} \times 100$$

The quantity of curcumin loaded in nanoparticles was calculated by deducting the amount recovered in the methanol fraction from the total amount of curcumin added.

The in vitro release of curcumin was carried out as follows. The curcumin-loaded nanoparticle was incubated at 37 °C with gentle agitation. The sample was centrifuged at 12000*g* for 20 min, to remove any unbound curcumin. The unbound curcumin, settled as pellet, was carefully collected and dissolved in methanol. Its absorbance at 425 nm was checked to calculate the amount of unbound curcumin. Similar experiments were carried out in acidic conditions (pH 2.0 with 0.9% NaCl).

The percentage of curcumin released was arrived at from the formula

$$\text{release (\%)} = \frac{[\text{curcumin}]_{\text{rel}}}{[\text{curcumin}]_{\text{tot}}} \times 100$$

where $[\text{curcumin}]_{\text{rel}}$ is the concentration of curcumin released in 24 h and $[\text{curcumin}]_{\text{tot}}$ is the total amount of curcumin entrapped in the nanoparticle.

Statistical Analysis. Data are presented as means ± standard deviations. For all of the measurements, a minimum of 3−4 replicates was taken for data analysis. Using the software Origin 6.1, all of the values were averaged and plotted.

RESULTS AND DISCUSSION

Stability of Curcumin. The effect of βLG on the stability of curcumin was followed in buffer at pH 7.0. The half-life of curcumin in buffer is 30.8 min. In the presence of βLG, the half-life of curcumin increased by 6.7 times to 206 min, indicating the protection of curcumin from hydrolytic degradation. Curcumin degrades rapidly under physiological conditions in vitro (*19*). Binding of curcumin to proteins may help in improving solubilization and arresting the degradation. The binding of curcumin to βLG delayed its hydrolytic degradation. α_{S1}-Casein, bovine serum albumin, and human serum albumin have also been reported to help enhance the stability of curcumin in solution (*20*, *21*). Curcumin in fetal calf serum has good stability with a half-life of 8 h (*19*). Fetal calf serum has several uncharacterized proteins that could bind curcumin, although the major protein is serum albumin. The stability of curcumin in various proteins follows the order: fetal calf serum (half-life, 8 h; pH 7.2) (*19*) > bovine serum albumin (373 min, pH 7.2) (*20*) > α_{S1}-casein (340 min, pH 7.2) (*20*) > β-LG (206 min, pH 7.0) (current study). β-LG bound curcumin is stable (half-life of 206 min) as compared to free curcumin in aqueous medium (30.8 min). The stability of vitamin D3 bound to βLG is enhanced as compared to the free vitamin (*22*). Our results along with previous reports suggest that βLG protected curcumin from degradation and increased its half-life in aqueous solution. Investigation of the binding of curcumin to βLG is of interest as βLG is believed to act as a natural transporting molecule.

Fluorescence Measurements. Curcumin in buffer when excited at 430 nm was weakly fluorescent with a nonspecific, feeble, and broad spectrum centered at ∼530 nm. The addition of βLG to curcumin solution (10 μM) resulted in a shift in the emission maximum from ∼530 to 500 ± 1 nm and increased the relative fluorescence intensity (**Figure 1A**). The fluorescence intensity increased linearly with an increase in the βLG concentration until the concentration ratio of curcumin−βLG reaches 1:4 (**Figure 1B**). The change in fluorescence intensity at 500 nm was recorded, and the data were plotted according to eq 1 (**Figure 1C**). The binding constant of curcumin to βLG at 25 °C, pH 7.0, is estimated to be $1.1 \pm 0.1 \times 10^5\ \text{M}^{-1}$. The fluorescence of curcumin, which depends on the polarity of the environment, showed a solvent-dependent shift in the emission maximum. A shift in the emission maximum from longer to shorter wavelength and increased fluorescence intensity indicated the movement of curcumin from a polar to a less polar environment (**Figure 1A**). This observation is in good agreement with the previous reports on binding of curcumin with proteins such as α_{S1}-casein and serum albumin. The binding of curcumin to α_{S1}-casein and human serum albumin exhibits emission maximum at 510 and 500 nm, respectively (*20*, *23*). The shift in the emission maximum along with an increase in the fluorescence intensity indicated that the binding of curcumin was hydrophobic in nature.

Fluorescence Quenching Measurements. Curcumin in buffer, although weakly fluorescent when excited at 430 nm (emission maximum at ∼530 nm), does not fluoresce when excited at 295 nm. However, in the presence of βLG, a new fluorescent peak was observed around 500 nm, reminiscent of the emission spectrum of curcumin when excited at 430 nm (**Figure 2A**). The addition of incremental aliquots of curcumin resulted in a gradual decrease in the fluorescence intensity centered at 335 ± 1 nm. The second fluorescent peak centered at 500 nm corresponds to the characteristic spectral region of curcumin. An isoemissive point was observed at 446 nm. The binding parameter was calculated by analyzing the quenching data. The inset of **Figure 2A** shows the mass action plot from which the binding constant is calculated to be $1.04 \pm 0.1 \times 10^5\ \text{M}^{-1}$.

The appearance of a fluorescence band in the spectral region characteristic of curcumin (centered at 500 nm) may be due to the energy transfer between the donor residues (aromatic amino acids) of the protein and the acceptor (curcumin). βLG has two tryptophan and four tyrosine residues per monomer (*24*) that contribute to the fluorescence of βLG. On addition of curcumin to βLG, a decrease in the tryptophan fluorescence intensity along with a concurrent increase in the fluorescence intensity centered at 500 nm was observed. At 295 nm, there is selective excitation of tryptophan residues. Trp19 is in an apolar environment on the βA strand at the base of binding pocket lying between 3.0 and 4.0 Å from the guanidine group of Arg124, while the partly exposed Trp61 lies on the C−D loop near the entrance of the β-barrel and the Cys66−Cys160 bridge (*24*). Curcumin probably binds in an apolar environment closer to Trp19 quenching the emission of Trp19 without causing any shift in the emission peak, when excited at 295 nm (*24*). Palmitate and retinoic acid are reported

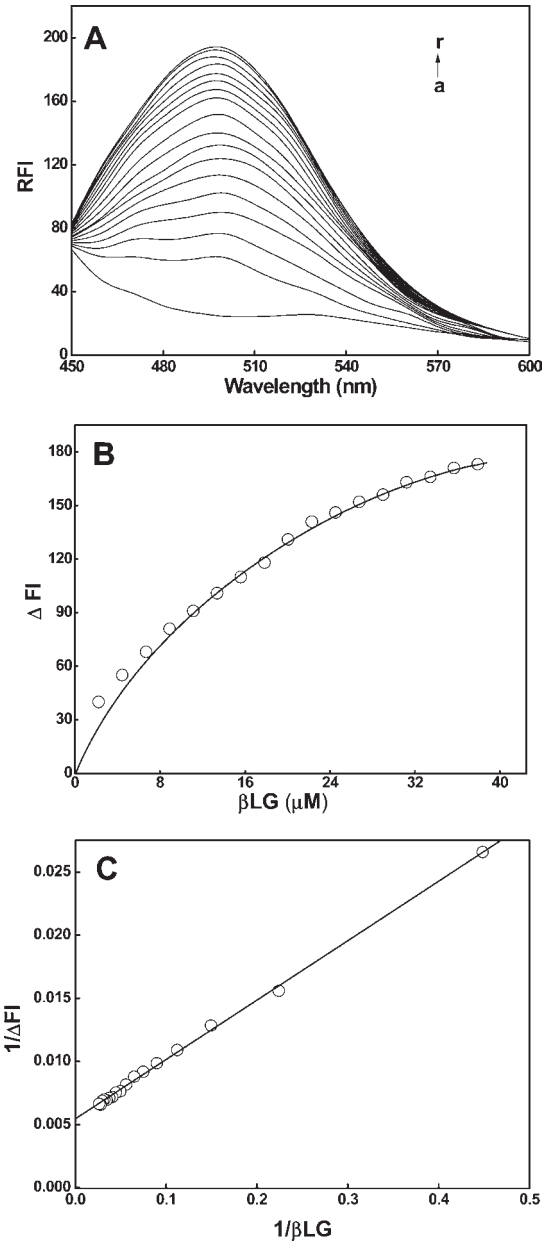

Figure 1. (**A**) Emission spectra of curcumin showing blue shift on binding to βLG. A 10 μM concentration of curcumin was titrated against an increasing concentration of βLG (a−r, 0−38 μM). The excitation wavelength was set at 430 nm, and excitation and emission slit widths were 5 and 10 nm. (**B**) Plot of fluorescence intensity as a function of βLG concentration. The maximum protein to ligand ratio for complex formation was 1:4. (**C**) Double reciprocal plot to calculate the binding constant as described in the Materials and Methods (R = 0.99).

to bind to βLG, with an association constant of 10×10^6 and 5×10^6 M^{-1}(*6*).

The effect of temperature on the association constant of curcumin to βLG was studied. **Figure 2B** shows the mass action plot for the curcumin−βLG system at different temperatures using an intrinsic quenching analysis. Thermodynamic parameters were calculated from the van't Hoff plot based on temperature dependence studies of the association constant in the range of 15−45 °C (**Figure 2B**, inset). The $\Delta S°$ value was 18.7 cal mol^{-1} K^{-1}, and the $\Delta G°$ value was −6.8 kcal mol^{-1} at 25 °C. Temperature dependence studies on the binding of curcumin to βLG revealed a decrease in the binding constant with the increase in temperature, suggesting the involvement of hydrophobic interactions.

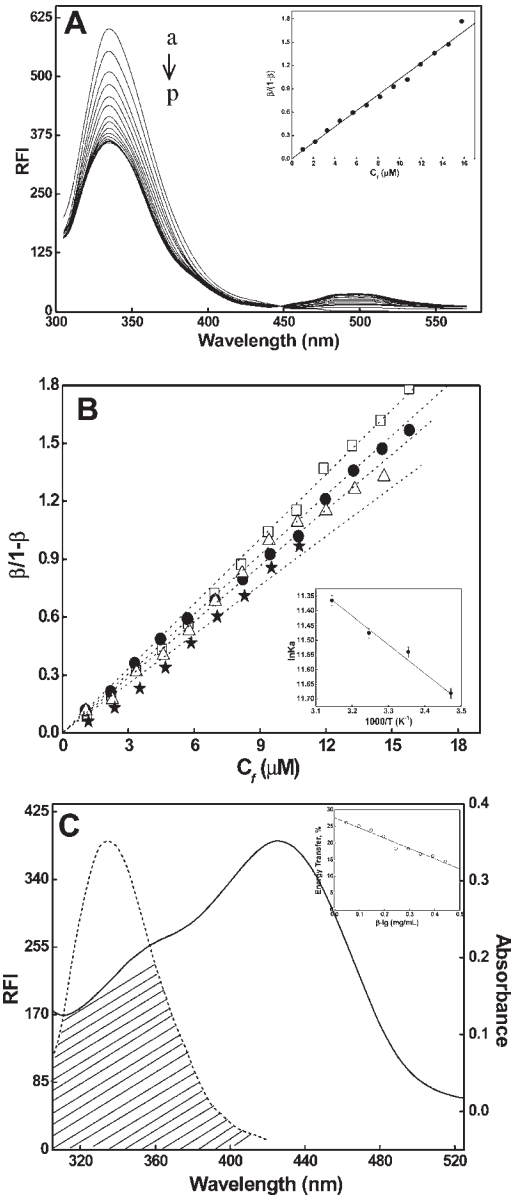

Figure 2. (**A**) Intrinsic fluorescence emission spectra of βLG and bound curcumin. The excitation wavelength was set at 295 nm, the protein concentration was fixed to 2.95 μM, and the temperature was maintained at 25 °C. Aliquots (2 μL) of curcumin were added in the concentration range 0−20.4 μM (a−p). Inset: Mass action plot; the slope gives the binding constant. (**B**) Mass action plot for the binding of curcumin to βLG at 15 (□), 25 (●), 35 (△), and 45 °C (★). Inset: van't Hoff plot; the error bars represent the mean and standard deviation of experiments in triplicate. (**C**) Overlap spectra for representing the Förster type resonance energy transfer between βLG and curcumin. Emission spectrum of βLG in buffer in the wavelength range of 305−400 nm; the excitation wavelength was 295 nm (- - -) and absorption spectrum of curcumin in buffer (—). The spectrum was taken immediately after adding curcumin to buffer. Inset: Plot of energy transfer between tryptophan of βLG (mg/mL) and curcumin.

The curcumin−βLG complex is accompanied by positive entropy changes, an indication of the binding process being entropically driven. Similar thermodynamic parameters are reported in relation to the binding of curcumin to α_{S1}-casein (*20*). On the basis of the characteristic signs of thermodynamic parameters for various interactions, it can be inferred that positive entropy changes are associated with hydrophobic interactions.

Table 1. Effect of pH on the Binding of Curcumin to βLG

pH	K_a (M^{-1})
5.5	1.1×10^3
6.5	2.4×10^4
7.0	1.1×10^5

FRET Measurements. To reveal the binding site of curcumin, resonance energy transfer experiments were carried out. FRET through Förster mechanism occurs when the emission spectrum of protein overlaps with the absorption spectrum of the ligand (*15*). **Figure 2C** shows the overlap spectrum. There was a fairly good overlap between the emission spectra of equimolar concentrations of βLG and the absorption spectra of curcumin (**Figure 2C**). The addition of curcumin resulted in the quenching of the fluorescence intensity, indicating Förster energy transfer. By integrating the spectra in the wavelength range 310−540 nm, the overlap integral J is computed to be 2.4×10^{-14} M^{-1} cm^3, and the energy transfer efficiency E is 0.27. The total percentage energy transfer is calculated by plotting the βLG concentration (mg/mL) versus the percentage energy transfer. The Y-intercept, which gives the energy transfer value, is 27% (inset of **Figure 2C**). The distance between the donor and the acceptor (r_o) is 32 Å, higher than 26.8 Å, the maximal critical distance (R_o). The higher value of r_o suggests a static type of quenching with nonradiation energy transfer between curcumin and βLG. The Förster distance (R_o) and the distance between the donor and the acceptor (r_o) obtained compare well with previous studies. Similar values of r_o and R_o have been obtained for βLG B variant and curcumin (r_o = 33.8 Å and R_o = 25.9 Å) (*25*). Because βLG contains two Trp residues, both have to be taken into account while calculating the efficiency of energy transfer, and the distance between the donor and the acceptor depends on the efficiency of energy transfer.

Effect of pH on the Binding of Curcumin to βLG. In the current study, experiments were carried out at pH 7, pH 6.5, and pH 5.5. At pH 5.5, βLG exists in closed conformation wherein the hydrophobic cavity is not accessible to ligand binding. At pH 7, βLG has an open conformation, allowing ligands to bind at the hydrophobic cavity. The effect of pH on the binding of curcumin to βLG was analyzed by determining its association constant. There is a precipitous decrease in association constant when the pH is decreased from 7.0 to 5.5 from 1.1×10^5 to 1.1×10^3 M^{-1} (**Table 1**). The decrease in the association constant with a decrease in the pH indicated inaccessibility of the ligand binding site to curcumin. Similarly, alkyl sulfonate ligands are reported to bind to βLG at pH 6.8, with no binding observed at pH 3.0 (*27*). At acidic pH, the EF loop (85−90) of βLG is in closed conformation, rendering the hydrophobic cavity inaccessible to the entry of ligands. With the increase in pH, the EF loop folds back, opening the gate for the access to the binding of ligands (*26*). At pH 7.0, the lid for the calyx is open with increased molecular volume/area (*26*), allowing curcumin to get into the hydrophobic pocket. This lends credence to our suggestion that curcumin may be binding at the hydrophobic pocket of the central calyx. Earlier studies of pH titration with βLG and palmitate have clearly shown that the binding site for palmitate is within the central calyx at neutral pH. At lower pH, the EF loop is in closed conformation and is stabilized by hydrogen bonds involving Ser116. Titration of Glu89 in EF loop at unusual high pH ~7.3 is due to deprotonation, which folds back the EF loop with a consequent solvent exposure of Glu side chain (*26*). Hence, opening of the EF loop appears to be a prerequisite for the binding of curcumin to βLG.

Curcumin-βLG Spectrum in the Presence of Palmitate. The binding site for palmitate in βLG is reported to be within the

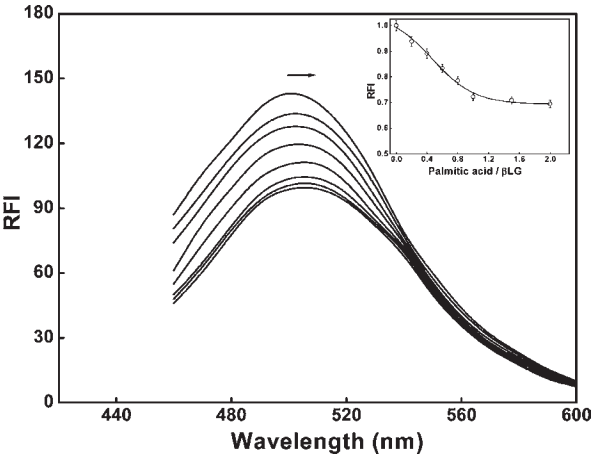

Figure 3. Effect of palmitate on the curcumin−βLG complex. Shift in the emission maximum of curcumin fluorescence on addition of palmitate from 500 to 505 nm (represented by arrow). The excitation wavelength was 430 nm, excitation and emission slit widths were set at 5 and 10 nm, and the concentrations of curcumin and βLG each were 10 μM. Inset: Plot of mole ratio of palmitate/βLG against normalized values of relative fluorescence intensity showing a decrease in fluorescence intensity of curcumin on the addition of palmitate.

β-barrel lined by hydrophobic residues (*17*). The effect of curcumin binding to βLG in the presence of palmitate was investigated. **Figure 3** shows the spectra of curcumin−βLG in the presence of palmitate. A solution containing an equimolar ratio of βLG and curcumin has an emission maximum at 500 ± 1 nm, when excited at 430 nm. The addition of aliquots of palmitate to the curcumin−βLG complex led to a red shift in emission maximum from 500 to 505 nm, with a simultaneous decrease in the fluorescence intensity of curcumin. The inset of **Figure 3** shows the mole ratio plot of [palmitate/βLG] against the normalized values of fluorescence intensity. The intensity decreased until the ratio of protein to palmitate reaches 1, with no perceptible decrease in the intensity thereafter. The red shift in emission maximum along with the decrease in fluorescence intensity indicated an alteration in the binding environment of curcumin. X-ray crystallographic studies have shown the binding of palmitate to the central cavity of the βLG, formed by eight antiparallel β-strands (*17*). Hydrophobic interactions are the predominant contributing factors for the affinity of fatty acids to βLG. Even though the binding constant (K_a) of palmitate to βLG is high when compared to the binding constant of curcumin to βLG, there was no significant decrease in the fluorescence intensity, even with excess addition of palmitate to the curcumin−βLG solution.

Binding of Curcumin with Denatured βLG. Titration of curcumin against denatured βLG was carried out at neutral pH, and the fluorescence spectra were recorded. The fluorescence emission maximum was 505 nm, indicating a +5 nm shift relative to the curcumin bound to native βLG. The change in fluorescence intensity at 505 nm was used for calculating the binding constant (data not shown). The binding constant of curcumin with denatured βLG was found to be $7.0 \pm 0.2 \times 10^2$ M^{-1}, which is very low as compared to that of native βLG. The low binding observed may be attributable to the nonspecific binding of curcumin to denatured βLG. In the presence of denatured βLG, curcumin (when excited at 430 nm) exhibits an emission maximum at 505 nm attributable to the loss in βLG structure. Conformational changes in the structure of βLG are extensive at the transition temperature of ≥70 °C (*28*). The D strand in the calyx participates in unfolding during thermal denaturation, resulting in the diminishment of calyx binding ligands such as palmitate and retinol (*29*).

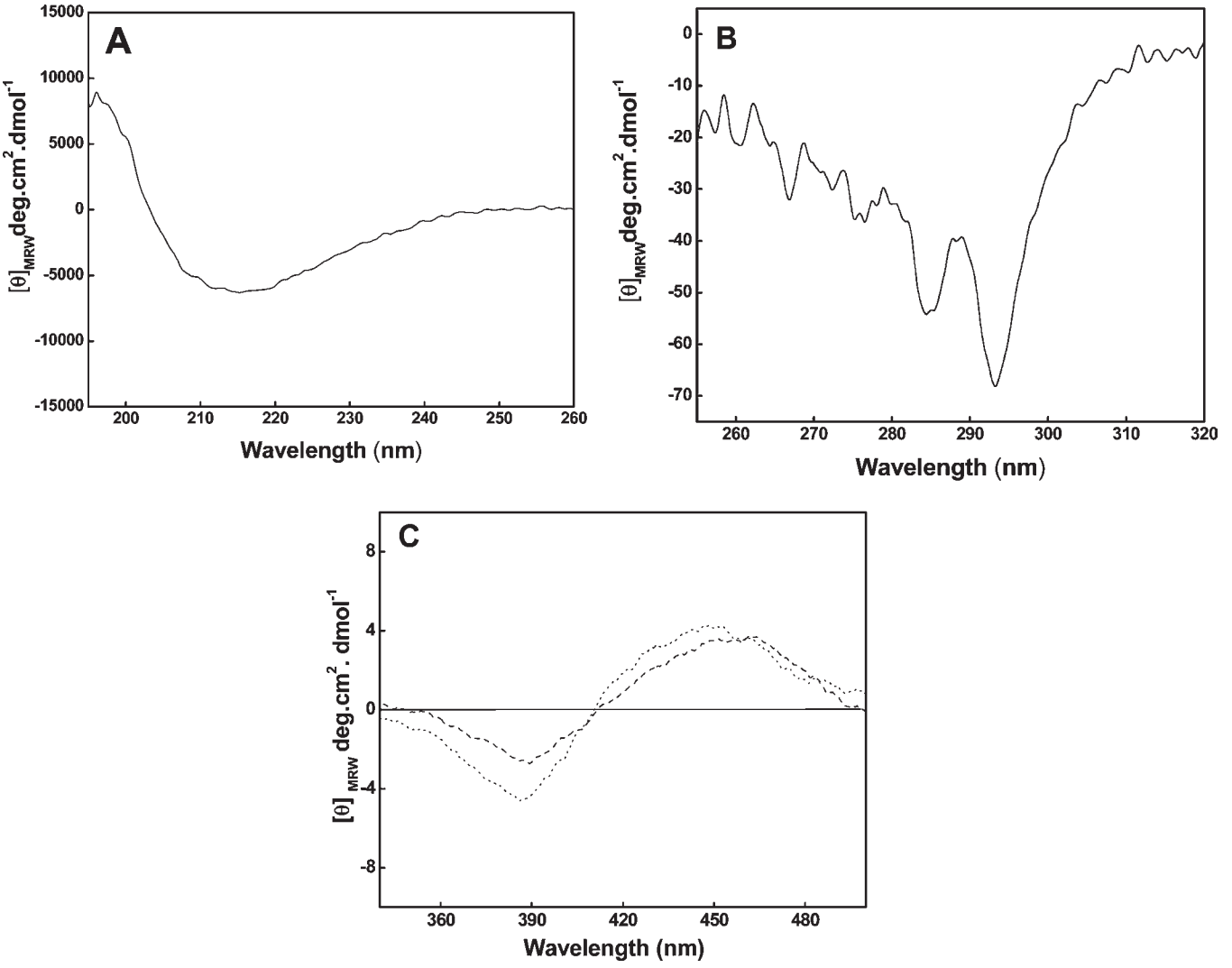

Figure 4. CD measurements of curcumin bound to βLG. CD spectra were recorded on a Jasco J-810 spectropolarimeter. An average of three scans at a speed of 10 nm/min, with a bandwidth of 1 nm and a response time of 1s, were recorded. The path length of the quartz cell was 1 mm for far UV and 10 mm for near and visible range. (**A**) The far UV spectrum of βLG was recorded in the wavelength range 195−260 nm. The concentration of the βLG is 0.2 mg/mL. (**B**) The near UV CD spectrum of βLG was recorded in the wavelength in the range 320−260 nm. The concentration of the protein is 1 mg/mL. (**C**) Induced CD spectrum of curcumin after binding to βLG recorded in the wavelength range 320−500 nm. The concentrations of curcumin were 7.5 (- - -) and 15 μM (· · ·), and βLG is 1 mg/mL. Tris HCl buffer (50 mM), pH 7.4, is used in all of the CD measurements. All of the readings were taken at 25 °C.

Therefore, heat treatment averts the binding of ligands to the central calyx.

CD Spectral Studies. The far UV CD spectrum of native βLG in 50 mM Tris HCl at pH 7.0 revealed a broad band with minimum at ~215 nm (**Figure 4A**) characteristic of the presence of a prominent β-structure. The near UV CD spectrum showed two sharp negative bands centered at ~292 and ~284 nm with two smaller bands located at ~277 and ~266 nm (**Figure 4B**). Curcumin did not exhibit any CD bands in aqueous solution. On interaction with βLG, it becomes asymmetric, resulting in the appearance of induced bands in the visible region of 350−500 nm. The CD spectra of curcumin in presence of βLG are given in **Figure 4C**. The induced negative and positive bands were observed at ~389 and ~450 nm, respectively. There are many examples of induced band formation for curcumin bound to protein (*20*, *23*, *30*). No change in the secondary and tertiary structure was observed on addition of curcumin to βLG. In the case of the βLG B variant, curcumin is shown to induce change in the helix and random coil content at pH 6.4 (*25*). However, the authors concluded that the changes observed are not significant and reflect conformational adjustments (*25*).

HPLC Measurements. To study the effect of curcumin on monomer−dimer equilibrium of βLG, gel permeation HPLC has been carried out. The HPLC profile shown in **Figure 5** shows that native βLG eluted at 23.5 min. Native βLG, in the presence of an equimolar concentration of curcumin, has an identical profile as βLG alone. Heated, denatured βLG when injected to the HPLC column eluted in two major peaks; the larger peak had a retention time of 19.6 min, indicative of aggregation. Similarly heated βLG, in the presence of an equimolar concentration of curcumin, exhibited a major peak eluting at 23.5 min as well as a small peak at 19.6 min. As compared to native βLG, the peak area of heated βLG in presence of curcumin is reduced by 8%. Thus, aggregation of βLG, in the presence of curcumin, is either prevented or delayed.

Native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE analysis of the denatured βLG, in the presence and absence of curcumin, under nonreducing and reducing conditions, did not reveal any difference in the band pattern between the control and the sample containing curcumin. Curcumin neither affected the state of association of βLG nor did it alkylate the cysteine residues (data not shown).

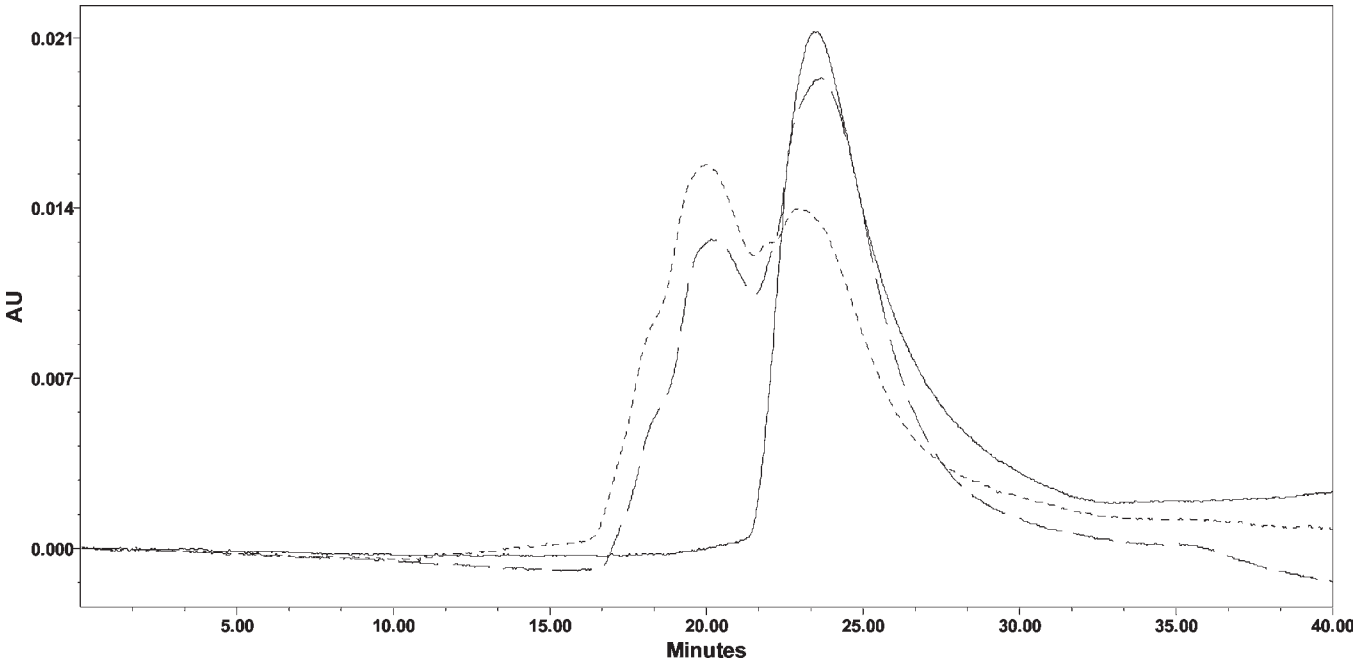

Figure 5. HPLC profile of βLG native (—), heated βLG (· · ·), and heated βLG containing curcumin (— —). The concentration of βLG is 1 mg/mL in 20 mM phosphate buffer, pH 6.6. The solution after heating to 85 °C for 10 min was cooled immediately. Gel filtration was performed using a TSK-Super SW2000 (4.6 mm $\times$300 mm, 4.0 μm) column on a Waters HPLC system (Waters, Milford, MA), equipped with a 1525 binary pump and Waters 2996 photodiode array detector. The column was equilibrated with phosphate buffer, and 20 μL of the above sample was injected. The sample was eluted isocratically in the same buffer. The flow rate was maintained at 0.2 mL/min at 25 °C. Detection was at 280 nm.

Heating of βLG at (65 °C) neutral pH resulted in the formation of aggregates due to scrambling of the disulfide linkages inside the protein core involving the free Cys121 (*31*, *32*). The appearance of a weak band is reported on heating at 65 °C for 45 min, corresponding to the dimer (*33*). The band corresponding to trimer is seen only after heating at 65 °C for 4 h. In the current study, βLG, in the absence or presence of curcumin, was heated at 85 °C for 10 min, cooled in an ice bucket, and analyzed by HPLC, native PAGE, or SDS-PAGE (under reducing or nonreducing conditions). The absence of any band in native PAGE and SDS-PAGE could mean that the aggregates were not detected with coomassie blue staining, considering the short time of heating. However, the aggregates could be detected by HPLC at 19.6 min. The aggregated proteins were not characterized. Curcumin might be providing a hydrophobic surface to Cys121, which may delay the aggregation process rather than preventing it. Curcumin is reported to irreversibly inhibit thioredoxin reductase by alkylating the cysteine and selenocysteine, present in the active site of the enzyme (*34*).

Visualization of the Binding Site: Docking Studies. Results of spectroscopic studies and effect of pH indicate that curcumin probably binds to βLG at the central calyx. On the basis of the experimental data, computational docking studies were performed to understand the binding site location and mode of binding of curcumin to βLG. The docking of curcumin to palmitate deprived βLG was investigated. Palmitate is known to bind to the central calyx of the βLG molecule (*17*). The representative build derived from the best pose with the minimal binding energy of -12.60 kcal mol^{-1} is shown in **Figure 6A**. The size of the calyx was found to be large enough to accommodate curcumin, which agrees with the spectroscopic data. No poses of the binding of curcumin within the surface hydrophobic patch on βLG were obtained. A careful inspection of the binding site (**Figure 6B**) suggested the closer contact of methoxy phenyl moiety of curcumin with the aromatic amino acid residues. The total number of hydrophobic contacts made by curcumin with the protein is 21. The central calyx of βLG is lined by hydrophobic amino acid residues that are gated by the protonation/deprotonation of Glu89 of the EF loop. The effect of pH on the binding of curcumin and the binding measurements with denatured βLG indicated the lower binding affinity of curcumin to βLG with a decrease in pH, pointing to the restriction in entry and binding of curcumin to the internal cavity of βLG. Within the van der Waals contact, the curcumin molecule is lined by hydrophobic residues such as Ile, Leu, Val, and Met that line the wall of the calyx and Phe105 in an orientation suitable to establish a $\pi-\pi$ interaction with the phenolic ring of curcumin. Lys60 is in the near vicinity of the methoxy group of curcumin. Lys60 and Lys69 are known to be involved in hydrogen bonding with palmitate (*17*). Pro38 is in contact with the hydroxyl group of curcumin. Pro38 is known to make contact with the 3-hydroxy group of cholesterol (*5*). Our results suggested the binding of curcumin to βLG predominantly by hydrophobic contacts within the calyx.

Nanoparticle Preparation and Measurements of In Vitro Release of Curcumin from βLG Nanoparticle. βLG, a major whey protein that constitutes 10−15% of the total proteins in bovine milk, exhibits a good emulsifying property due to its amphiphilic nature (*12*). βLG is proposed to be utilized for encapsulation as well as controlled release of bioactive compounds by preparing hydrogels or nanoparticles based on its superior gelling property (*8*) and its ability to be a natural carrier of many biochemically important hydrophobic compounds, such as retinol and its derivatives, palmitate, cholesterol, and vitamin D (*5*).

Nanoparticles of curcumin encapsulated in βLG have been prepared, and the solubility and in vitro release of curcumin from βLG nanoparticle have been studied. The morphology of the prepared nanoparticles of βLG is analyzed using SEM. The SEM images in **Figure 7A**,**B** correspond to βLG alone and curcumin encapsulated by βLG particles, respectively. **Figure 7C** shows the graphical representation of the size distribution of the βLG nanoparticles. The particles are polydispersed with the average size being 142 $\pm$ 5 nm. They are spherical in shape, and no

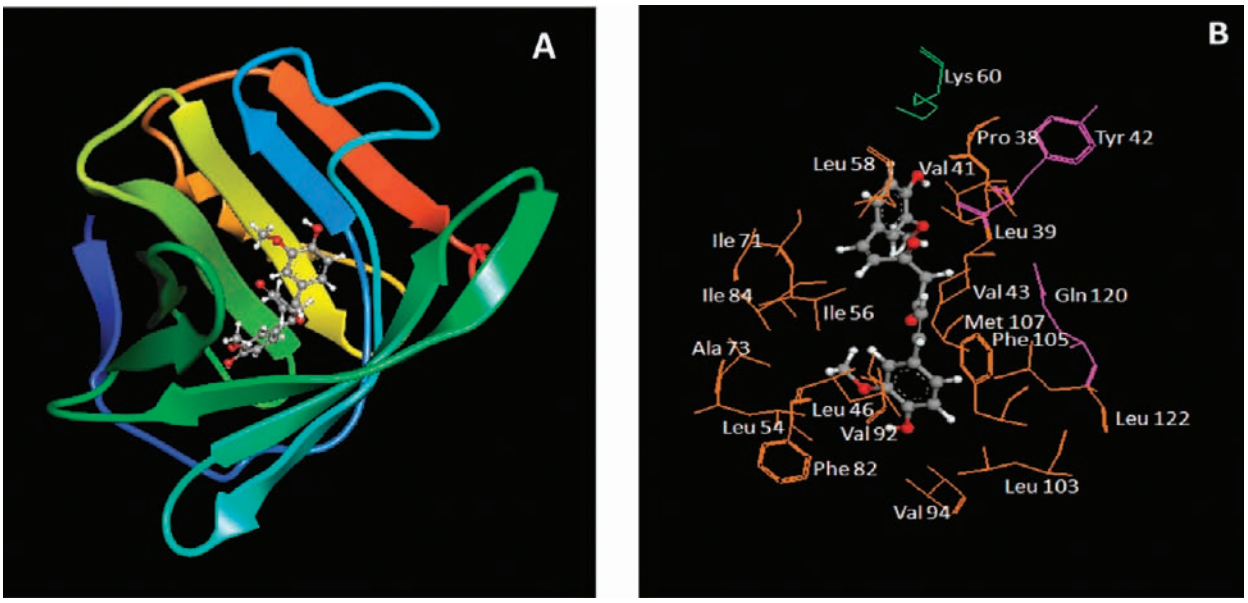

Figure 6. Molecular docking studies. (**A**) Cartoon ribbon model structure of βLG showing the binding of curcumin (gray) to central calyx. (**B**) Amino acid residues surrounding the curcumin molecule. The orientation from the original structure (PDB ID: 1B0O) has been changed to clearly view the binding of curcumin to the βLG calyx.

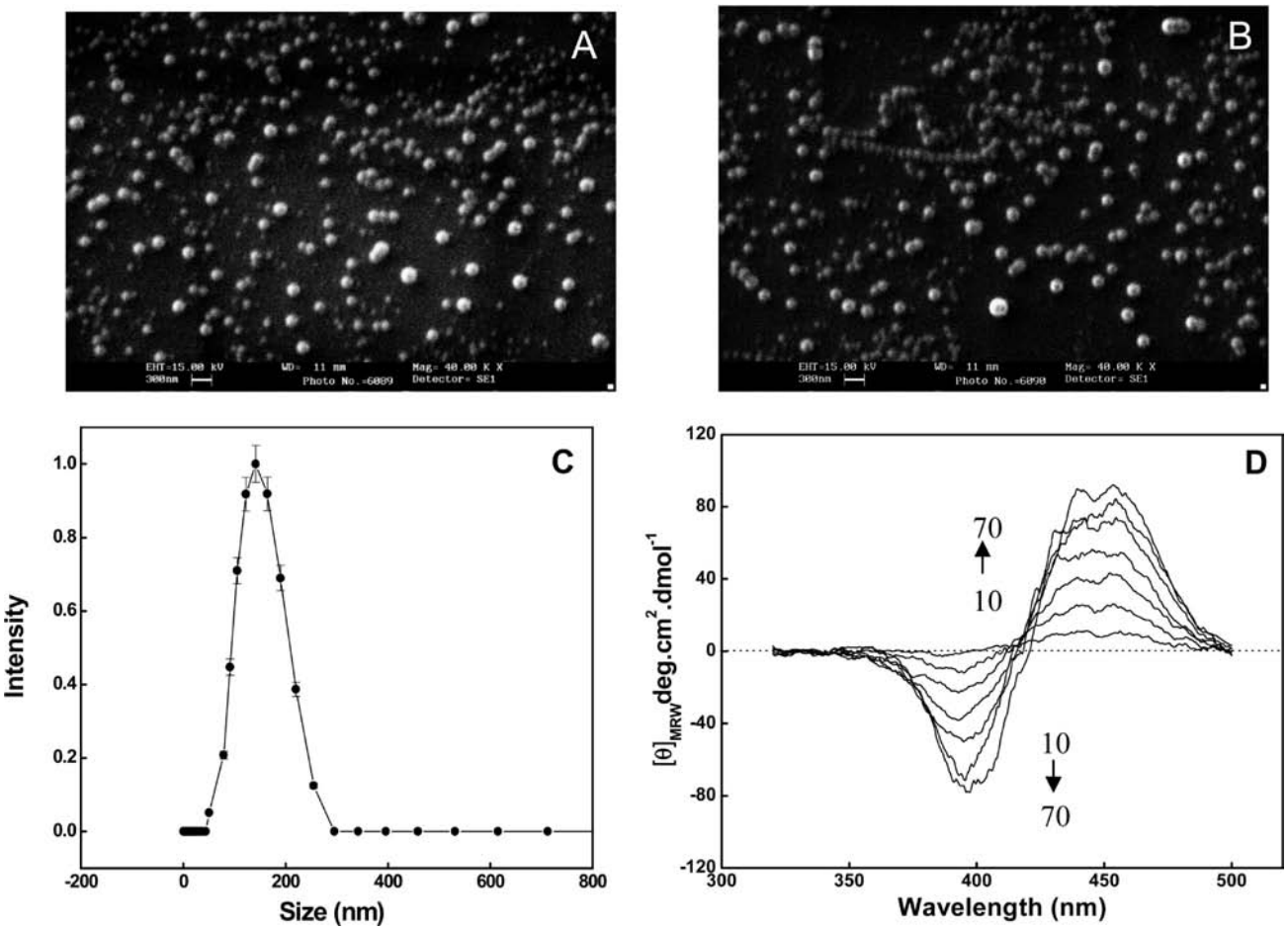

Figure 7. Scanning electron microscopic photograph of (**A**) βLG nanoparticle and (**B**) βLG nanoparticle loaded with curcumin. The sample was vacuum-dried and coated with gold before viewing under a microscope. (**C**) Graphical representation of particle size distribution of curcumin encapsulated in βLG nanoparticle at pH 7.0. The average size of particles was 142 ± 5 nm. Similar results were obtained with βLG nanoparticle. The error bars represent the mean and standard deviation of experiments in duplicate. (**D**) Induced CD spectra of curcumin encapsulated in βLG nanoparticle. The concentration of βLG nanoparticle is 1 mg/mL in 50 mM Tris HCl buffer, pH 7.0, and the concentration of curcumin is from 10 to 70 μM (the arrow mark indicates the increasing concentration of curcumin).

significant physical change was observed, relative to control (βLG). The average size of βLG nanoparticle is reported to be in the range of ∼60 nm (*12*). The size of the particles decreases with an increase in pH (*12*). Encapsulation of curcumin in the nanoparticles was confirmed by the appearance of fluorescence spectrum at 505 nm, when excited at 430 nm.

The induced CD spectra of curcumin encapsulated in βLG nanoparticle is given in **Figure 7D**. An induced positive band and a negative band at ∼450 and 395 nm were observed, respectively. There was an increase in the two induced bands concomitant with a rise in the curcumin concentration in the range 10−70 μM, confirming the encapsulation of curcumin. The solubility of curcumin in aqueous solution is very low (30 nM) (*2*). The solubility of encapsulated curcumin increased significantly to ∼625 μM. In surfactant micellar solution, the solubility of curcumin is enhanced to ∼740 μg/mL, in contrast with the solubility of curcumin in aqueous solution (*35*). The encapsulation efficiency of curcumin within the βLG nanoparticle is found to be >96%.

Curcumin was released from βLG nanoparticles up to ∼16%, under in vitro conditions after 24 h. In vitro release kinetics was analyzed to study the release of curcumin from βLG nanoparticles at neutral pH. The in vitro release of curcumin from βLG nanoparticles was found to follow zero order kinetics. The zero order release constant, k_o, was 1.17 M s^{-1}. Overall, our results indicated that encapsulation of curcumin with βLG enhanced solubility with a slow release of curcumin in vitro. In vitro release of curcumin from βLG nanoparticles was also checked under acidic conditions (pH 2.0). No release of curcumin was observed, over a period of 48 h, from the nanoparticles. The concentration of protein and ligand affects the encapsulation efficiency as reported by Somchue et al. (*36*). To prolong the release of α-tocopherol in simulated intestinal conditions, coating with alginate was carried out as there was immediate release of α-tocopherol in simulated gastric conditions (*36*). Nearly 51% of curcumin is released from the alginate−chitosan−pluronic acid composite particles under neutral pH conditions. The release kinetics in this case obeys the power law with the "anomalous transport" being the release mechanism (*37*).

In conclusion, we report the detailed spectroscopic study on the interaction of curcumin with βLG and preparation of curcumin encapsulated in βLG nanoparticles. Curcumin probably binds to the central hydrophobic cavity of βLG surrounded by hydrophobic amino acids forming a 1:1 complex. Enhancement of the solubility and stability of curcumin, when bound to βLG, may be helpful in improving the bioavailability of curcumin in vivo. Curcumin-bound βLG can be an effective carrier for curcumin as both are food components.

ABBREVIATIONS USED

βLG, β-lactoglobulin; HPLC, high-performance liquid chromatography; SEM, scanning electron microscopy; FRET, fluorescence resonance energy transfer.

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