Biophysical Chemistry 165–166 (2012) 13–20

Contents lists available at SciVerse ScienceDirect

Biophysical Chemistry

journal homepage: http://www.elsevier.com/locate/biophyschem

Highly efficient immobilization of beta-lactoglobulin in functionalized mesoporous nanoparticles: A simple and useful approach for enhancement of protein stability

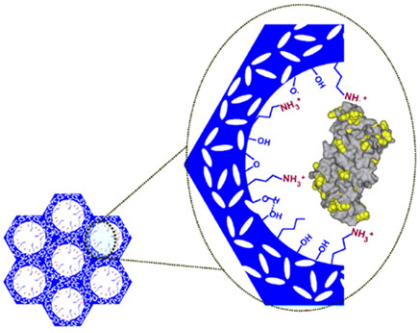
Mojtaba Falahati [a,b], Ali Akbar Saboury [a,*], Abbas Shafiee [c,**], Seyed Mahdi Rezayat Sorkhabadi [b], Ehsan Kachooei [a], Leila Ma'mani [c], Thomas Haertlé [d]

[a] Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[b] Department of Biology, Faculty of Advance Science and Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran
[c] Department of Medicinal Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran
[d] UR 1268 Biopolymères Interactions Assemblages, INRA, équipe Fonctions et Interactions des Protéines Laitières, B.P. 71627, 44316 Nantes Cedex 3, France

HIGHLIGHTS

- Immobilization of BLG onto the [n-$PrNH_2$-KIT-6].
- Highly absorbed amount of BLG.
- Higher stability of immobilized BLG.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 January 2012
Received in revised form 25 February 2012
Accepted 27 February 2012
Available online 4 March 2012

Keywords:
KIT-6
β-lactoglobulin-B
Immobilization
Functionalization
Stability
Aggregation

ABSTRACT

The immobilization of β-lactoglobulin-B (BLG-B) onto the amine-functionalized KIT-6 [n-$PrNH_2$-KIT-6], which has average pore diameter around 6.5 nm, was studied. [n-$PrNH_2$-KIT-6] proved to be highly effective agent for BLG-B adsorption. UV–visible spectroscopy studies demonstrated that the immobilized BLG-B was less prone to thermally induced aggregation than the free protein. Circular dichroism (CD) spectra of free and immobilized BLG-B were recorded and significant differences in both the backbone and aromatic regions of the spectra were observed upon thermic stress. The obtained results showed that structural elements of the immobilized BLG-B are kept strongly together, making the protein more resistant to heat denaturation. The melting temperatures of the free and immobilized BLG-B were measured by far-UV CD, which showed 19 °C higher heat resistance of the immobilized BLG-B compared with its free form. Acrylamide quenching of fluorescence of free and immobilized forms of BLG-B as a function of temperature revealed that the immobilized BLG-B was more resistant to Trp quenching. Therefore immobilization of BLG-B onto [n-$PrNH_2$-KIT-6] is accompanied by favorable structural stability of BLG-B in the confined space.

1. Introduction

Immobilization of proteins on solid supports sometimes can be an efficient technique to increase their structural stability. It offers several advantages such as improved structural and operational stability, as well as the resistance to high temperatures, extreme pH, and organic (co)-solvents.

* Corresponding author. Tel.: +98 2166 409517; fax: +98 2166404680.
** Corresponding author. Tel.: +98 21 66406757; fax: +98 2166461178.
E-mail addresses: saboury@ut.ac.ir (A.A. Saboury), shafieea@sina.tums.ac.ir (A. Shafiee).

0301-4622/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2012.02.006

Ordered mesoporous materials were discovered in 1992 by Beck et al. [1]. Their potential for protein immobilization was explored in 1996 by Diaz and Balkus [2]. Thanks to their well-defined pore structure, which is characterized by a narrow distribution of pore diameters and high internal surface area they are able to absorb many different macromolecules [2]. Since then, various proteins including cytochrome c, trypsin, lysozymes, lipases, α-chymotrypsin and so many others have been successfully immobilized on ordered mesoporous materials belonging to the Mobile Crystalline Material (MCM) and Santa Barbara Amorphous (SBA) families [3–8].

However, protein diffusion and migration within the mesoporous materials used in previous studies was hampered by a pore structure, small size, and low pore volume. Three dimensional cubic mesoporous silica nanoparticles (MSN) with large pore sizes overcome these limitations, and therefore they are very promising candidates for potential applications in protein immobilization [7,9–11]. Klietz et al. developed a new family of cubic mesoporous Ia3d silica called KIT-6 using a mixture of P123 (Pluronic (PEO)20-(PPO)70-(PEO)20) and n-butanol [12]. KIT-6 features large, readily tunable pores and regulated pore volume, high specific surface area and hydrothermal stability, making it a perfect candidate for protein adsorption. Due to weak interactions between the protein and material surfaces, these solid supports cannot effectively protect the immobilized bio-molecules from leaching. One approach to inhibit the amount of protein leaching is to decrease the pore opening at the external surface by silanation. Another applicable and useful approach is the functionalization of the inorganic surface in order to change the physical and chemical properties of mesoporous materials. A specific example is the introduction of organic moieties with pendant-attached chains on the solid surface, which could result in additional electrostatic attractions or repulsions, and/or increased hydrophobic or hydrophilic interactions between mesoporous silica nanoparticles and adsorbed biological macromolecules [13]. The key point is that the normally weak surface-macromolecule interactions can be made stronger in practice by immobilization of the protein on such "inorganic–organic hybrid materials".

BLG-B is a well-characterized small globular protein (MW = 18,300 Da) from the milk whey of ruminants and other mammals [14]. At ambient temperature and at the pH equal or higher than 7 it is prevalently dimeric. In addition to hydrophobic, ionic and hydrogen-bond interactions between the peptide chains stabilizing a favored protein conformation, BLG-B is stabilized by two disulphide bridges. In native BLG-B the two stated disulphide bridges (Cys66–Cys160 and Cys106–Cys119) and the free thiol group (Cys121) are located in a hydrophobic core of globulin, and are inaccessible to the polar medium [15–17].

A number of studies have described in detail the heat denaturation of β-lactoglobulin in defined environment with the use of optical methods [18–25]. Raising the temperature up to 55 °C shifted the equilibrium (monomer ↔ dimer) to monomer. As temperature was increased to around 65 °C a slight conformational change of the monomers was observed, leading to what has been called the "molten globule state", in which native-like secondary structure content is conserved but the tightly folded protein interior is destabilized. At 85 °C, larger secondary and tertiary structural changes were observed. These large-scale changes will therefore induce thiol/disulphide exchange reactions and exposure of hydrophobic residues of the protein, causing aggregation of the monomers [26].

Heat treatment is an important process during milk processing in the manufacture of most dairy products. Heating during pasteurization is used to increase the safety of the food products [27]. Such heat treatments cause structural changes in the native bovine BLG-B [28], and may alter its properties [29].

Several possible functions have been reported for BLG. BLG contains a rich source of Cys, an essential amino acid that stimulates glutathione synthesis in the liver. Glutathione is an anticarcinogenic tripeptide for protection against intestinal tumors [30]. It has also reported that BLG-B may be used as a carrier molecule for several anti-tumor compounds [31].

Several recent reports have shown promising results in improving mesoporous silica nanoparticles biocompatibility and cell membrane permeability [32–37]. Therefore structural stability enhancement of BLG-B by means of its immobilization on the mesoporous materials can open a new avenue for the development of anti- cancer and drug delivery systems.

In other words the enhancement of BLG-B structural stability by its immobilization on mesoporous materials in severe denaturing conditions may be of considerable interest.

In this paper the immobilization of β-lactoglobulin-B (BLG-B) with an average hydrodynamic radius of 2 nm [38] onto the amine-functionalized KIT-6 [n-PrNH$_2$-KIT-6], which has average pore diameter around 6.5 nm, was studied. The aim of this study was based on the investigation of the structural changes of free and immobilized BLG-B after heat denaturation. These amine-functionalized mesoporous silica nanoparticles (MSN) were prepared using a non-ionic surfactant and were fully characterized by spectroscopic, adsorptive, nitrogen porosimetry and thermal techniques in our previous paper [39]. In the present study, UV–visible spectroscopy was used to detect the difference in absorbance at 360 nm (which is a measure of the extent of aggregation), while far and near-UV circular dichroism was applied in monitoring the changes of secondary and tertiary structure, respectively. Fluorescence spectroscopy also was used to investigate tertiary structure changes of free and of immobilized BLG-B.

2. Material and methods

2.1. Materials

Bovine BLG-B was purchased from the Sigma. All other materials and reagents were of analytical grade. All solutions were made in double-distilled water.

2.2. Preparation of BLG-[n-PrNH$_2$-KIT-6] composites

Amine-functionalized mesoporous nanoparticle (100 mg) was added to BLG-B solution (10 ml of 10 mg/ml) and stirred for 24 h at 4 °C in phosphate buffer (20 mM, pH 7.8). The supernatant was separated from the solid materials by centrifugation at 6000 rpm for 5 min.

The protein content of the supernatant was defined spectrophotometrically measuring absorption at 278 nm with the extinction coefficient of 17600 M^{-1} cm^{-1}. Thus, the amount of immobilized BLG-B could be simply estimated from the difference between the concentration of the BLG-B before and after adsorption. In this case enzyme molecules linked on external surface of the particles would remain there instead of being removed. However, these molecules should be easily desorbed upon incubation in aqueous medium. Therefore the resulting solid was then washed three times by the same buffer during 15 min and separated by centrifuging to remove the BLG-B molecules linked on external surface of the nanoparticles from the retained within the silica. Therefore we can ignore the possibility that the protein may just be immobilized onto the surface of the material and also with other evidence (Supplementary data) thus we can conclude that it has been immobilized inside the pores. Finally BLG-[n-PrNH$_2$-KIT-6] composites were air dried and stored at 4 °C for further structural experiments.

2.2.1. Assessment of leaching

The immobilized BLG-B in mesoporous re-suspended by stirring in a phosphate buffer pH = 7.8 for 2 h. The suspended solids were allowed to settle for 2 h. The content of BLG-B in the supernatant from above the

solid phase was measured using again the Beer–Lambert law to define the amount of BLG-B leached from the synthesized mesoporous silica nanoparticles.

2.2.2. UV–visible spectroscopy

The UV–visible absorption spectra were obtained using a UV-3100, Shimadzu, Japan. Turbidity was measured at 360 nm against a nanoparticles-phosphate buffer blank to detect formation of protein aggregates. Absorbance changes of free and immobilized BLG-B with the same protein content of 0.5 mg/ml were recorded by the UV–visible absorption as temperature was increased from 35 to 85 °C at the rate of 1 °C/min. All experiments were run in phosphate buffer (20 mM, pH 7.8). All spectra were background-corrected against buffer and [n-$PrNH_2$-KIT-6] solution to diminish the scattering of the beam by the silicate particles.

2.2.3. Circular dichroism (CD) spectra of BLG-B and BLG-B immobilized on [n-$PrNH_2$-KIT-6]

Circular dichroism spectra (CD) were acquired using an Aviv model 215 Spectropolarimeter (Lakewood, NJ, USA) equipped with a 1-cm path length quartz cuvette of 300 μl capacity. Changes in the far-UV (190–260 nm) and near-UV (260–340 nm) CD spectra of free and immobilized BLG-B over different temperatures ranges were monitored. Protein concentrations for both free and immobilized BLG-B was the same content, whereas for far-UV and near-UV CD spectral measurements it was 0.2 and 0.5 mg/ml in phosphate buffer of 20 mM at pH 7.8, respectively. For avoiding the scattering of the beam by the silicate particles, all spectra were background-corrected against buffer and [n-$PrNH_2$-KIT-6] solution. The obtained results were expressed as ellipticity (deg cm^2 $dmol^{-1}$) based on a mean amino acid residue weight (MRW) of 114 Da for BLG-B having the average molecular weight of 18.3 kDa.

2.2.4. Tm measurements

Heat denaturation curves were determined by monitoring the changes of CD absorbance at 218 nm with an Aviv model 215 Spectropolarimeter (Lakewood, NJ, USA) using a 1-cm cuvette. BLG-B samples at a concentration of 0.2 mg/ml (for both free and immobilized BLG) were dissolved in 20 mM potassium phosphate buffer, pH 7.8. The temperature of sample solutions was measured directly and raised linearly at a rate of 1 °C/min. The heating curves were corrected for an instrumental baseline which obtained by heating the buffer (20 mM potassium phosphate, pH 7.8 and mesoporous silica solution) alone.

2.2.5. Fluorescence spectroscopy

Fluorescence intensity quenching of Trp residues by acrylamide was monitored using a Hitachi spectrofluorimeter, MPF-4 model. The widths of the excitation and the emission slits were 10.0 nm and 5.0 nm, respectively. The tryptophan fluorescence spectra of the free and immobilized BLG-B (same content of 0.05 mg/ml) were measured in the presence of different concentrations of acrylamide (0–0.5 M). All experiments were carried out in the temperature range from 65 to 85 °C for both free and immobilized BLG-B. The experiments were performed in 20 mM phosphate buffer (pH = 7.8).

The measurements of heat denaturation were performed as follows: the samples were held at temperatures of 65, 75 and 85 °C, for 15 min. After 15 min heating each of the samples was cooled immediately to the room temperature. The emission spectrum (excitation at 295 nm) of each solution was measured between 300 and 470 nm and λ_{max} determined (F_0). Acrylamide was added to the sample to the final concentrations ranging from 0 to 0.5 M and the resulting fluorescence intensity quenching was determined (F). The data were analyzed using a Stern-Volmer plot (F_0/F versus acrylamide concentration).

All measurements were made in triplicate.

3. Results and discussion

Amine-functionalized KIT-6 ([n-$PrNH_2$-KIT-6]) nanoparticle as inorganic–organic hybrids could be promising candidates for the immobilization of BLG-B. The cubic mesoporous silica was fully characterized by X-ray diffraction (XRD), FT-IR spectroscopy, nitrogen adsorption-desorption isotherm, thermal gravimetric analysis (TGA), CHN elemental analysis and back titration [39], (Supplementary data).

3.1. Adsorption and leaching assessment

Protein molecules can be immobilized onto the pores of mesoporous materials by simply immersing the mesoporous material in the enzyme solution [2]. It has been observed that the immobilization of proteins on the purely siliceous mesoporous nanoparticles results in considerable leaching during reaction [7]. This is due to the weak interactions between the protein molecule and the surfaces of the mesoporous nanomaterials. Functionalization of the mesoporous solid mesoporous enhances the interactions between the immobilized protein and the solid surfaces. Our results also demonstrate the advantages of functionalization in decreasing the leaching of the enzyme from solid support.

The isoelectric point (pI) of BLG-B is around 4.5, and at higher pH it is charged negatively. This implies that [n-$PrNH_2$-KIT-6] has a strong adsorptive capacity for BLG-B. Otherwise, the pKa of amino groups is 9 and thus, at pH 7.8, the amine is protonized as $-NH_3^+$. Hence, thanks to electrostatic adsorption between BLG-B and the mesoporous material surface at pH 7.8 the amine groups (with positive charges) are potential candidates to be grafted on the KIT-6 surface for improving the efficiency of BLG-B immobilization.

We observed that amine-functionalized mesoporous silica nanoparticles ([n-$PrNH_2$-KIT-6]) had a great capacity for adsorption of BLG-B up to about 638 mg g^{-1} of support. This amount of immobilized BLG-B remains in agreement with the observed weight loss of 62.7% in the second step of TG curves for BLG-B-[n-$PrNH_2$-KIT-6] (Supplementary data).

After immobilization, the most important is to inhibit desorption of the protein from the mesoporous material. The strength of the interactions between protein molecules and the support can be tested by measurement of the leaching of the protein. Leaching tests were carried out in a phosphate buffer 20 mM, pH 7.8. The amount of protein leakage was confirmed by measuring the UV–vis spectrum after stirring the silica in a phosphate buffer pH = 7.8 for 2 h. After 2 h, no changes were observed in the supernatant UV–vis spectra, confirming that the BLG-B does not leach from the studied [n-$PrNH_2$-KIT-6]. Hence it may be concluded that BLG-B molecules are tightly adsorbed on the amine-functionalized mesoporous material. As a final step, we corrected the data against mesopourous phosphate buffer blank in order to avoid scattering. Since the leaching of the enzyme during 2 h (spectroscopic time scale) is essentially zero, we can be confident that the spectroscopic signal corresponds to immobilized protein.

3.2. UV–visible spectra studies

Protein aggregation is an inevitable consequence of cellular existence and is a significant factor in a variety of pharmaceutical and biotechnological processes. Decreasing protein aggregation in vivo is important to prevent a range of diseases from sickle-cell anemia to Alzheimer's disease [40,41]. Improvement of structural and technologic stability of proteins under extreme conditions is one of the ultimate goals of protein science. Since temperature is an important physical variable, a wide range of studies have been focused on elucidation of structure–stability relationship under heat stress [18–25,39].

The objective of this experiment is to identify the impact of immobilization on the thermal aggregation of BLG-B. We hypothesized that the immobilization of BLG-B onto the nanoparticles would influence its unfolding and the aggregation of the protein. UV–visible spectrophotometry has been extensively used as a sensitive monitor of subtle changes in protein conformation and aggregation [42]. Proteins in solution come in contact with each other because of a number of forces, such as oppositely charged subunits or hydrophobic residues, to form highly stable aggregates. Protein aggregation changes the functional and enzymatic properties of a protein.

That's why control of aggregate formation in severe conditions is the one of the interests of much current biotechnology. An absorption change at 360 nm is a well-known evidence for the turbidity and accordingly the presence of aggregates [43,44]. The aggregation of the free and immobilized BLG-B was monitored by measuring turbidity versus temperature. Aggregation changes of free and immobilized BLG-B measured at 360 nm is shown in Fig. 1. It was clearly shown that immobilization of BLG-B markedly suppresses the protein aggregation during heat denaturation. In case of immobilized BLG-B, the midpoint of aggregation, Ta, has increased from 71.1 ± 1 °C to 82.9 ± 1 °C (Fig. 1). It implies that the presence of additional electrostatic interactions between mesoporous surfaces and BLG-B results in improved structural stability of the protein and inhibits the formation of aggregate species. This experimental evidence suggests that a strengthening of the BLG-B molecule could be a simple and effective strategy to minimize the deformation of its structure reducing its heat unfolding.

Furthermore it is amply documented that the aggregation of proteins depends heavily on the intensity of protein–protein interactions. Hence any decrease of these contacts would prevent the aggregation. Protein immobilization on a mesoporous nanomaterial is a good strategy to reduce protein–protein interactions by changing the protein's microenvironment. In fact, the obtained results show that the heat-induced aggregation of protein is delayed by immobilization of BLG-B on the [n-PrNH$_2$-KIT-6].

3.3. Circular dichroism studies

Circular dichroism is an advanced method for monitoring secondary and tertiary structural changes in proteins, triggered by changes of temperature, pH and so on [45]. It has also been used for structural studies of immobilized enzymes onto the mesoporous materials [39,46,47]. The far UV–CD spectra for free and immobilized BLG-B from 25 °C up to 85 °C are shown in Fig. 2 (a, b) and the results of applying the CDNN software to the calculation of secondary structure content are summarized in Tables 1a and 1b. Fig. 2 (a, b) shows the

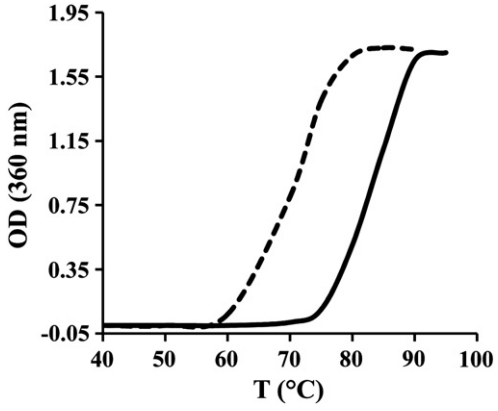


Fig. 1. Turbidity (OD_{360}) vs. temperature for free (— —) and immobilized BLG-B (—). BLG concentration was 0.5 mg ml^{-1} in 20 mM phosphate buffer, pH 7.8.

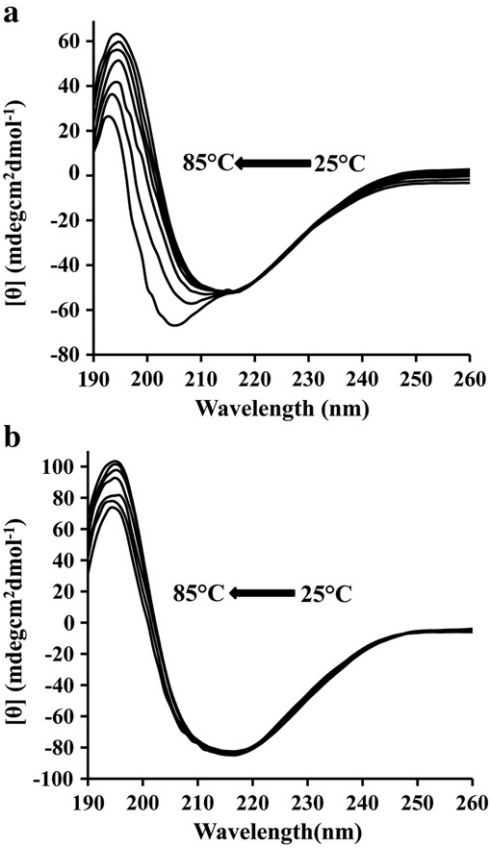


Fig. 2. Far-UV circular dichroism (CD) spectra of free (a) and immobilized BLG-B (b) measured at different temperatures from 25 to 85 °C in 20 mM phosphate buffer, pH 7.8.

CD spectrum of BLG-B, which has a strong minimum peak at 218 nm typical of predominant β-sheet. A comparison of the far-UV CD spectra of free (Fig. 2 (a)) and immobilized BLG-B (Fig. 2 (b)) reveals that elevating temperature leads to a considerable change in far-UV CD spectra of free BLG-B relative to immobilized BLG-B, indicating a more intense unfolding of free BLG. In case of free BLG-B the amount of β-sheet and α-helix decreased considerably compared to immobilized BLG-B. The present results therefore indicate an almost 10% and 15% loss of α-helix and β-sheet in the range of 25 to 85 °C in case of free BLG-B, respectively (Table 1a).

The X-ray crystal structure of BLG-B shows a three-turn helix to be the main secondary structural feature of protein [48]. Disruption of the three-turn helix could induce exposure of the free thiol, Cys-121, that is normally buried in a hydrophobic cleft. Around 65 °C the free thiol group becomes exposed and is able to form new intermolecular disulphide bridges [49]. The decrease in β-sheet amount in the temperature range studied can be explained by the destabilization of monomers, which form in the dimer an intermolecular β-sheet with its partner. However, the immobilized BLG-B exhibits a remarkable resistance to thermal denaturation in comparison with free BLG.

Table 1a
Typical structural changes of free BLG-B in raising temperature as shown by the analysis of CD spectra.

Temperature (°C)	β-Sheet (%)	Random coil (%)	α-Helix (%)	Turn (%)
25	36.4	34.7	12.0	16.9
35	35.4	34.6	12.3	17.7
45	35.2	34.9	12.1	17.8
55	34.7	35.1	11.9	18.3
65	34	35.4	11.8	18.8
75	32.5	36.6	11.4	19.5
85	31	37.7	10.8	20.5

Table 1b
Typical structural changes of immobilized BLG-B in raising temperature as shown by the analysis of CD spectra.

Temperature (°C)	β-Sheet (%)	Random coil (%)	α-Helix (%)	Turn (%)
25	36.5	31.7	12.5	19.3
35	36.5	31.7	12.5	19.3
45	36.7	32.5	12.3	18.5
55	36.8	30.7	12	20.5
65	36.4	32	11.9	19.7
75	35.7	32.3	11.7	20.3
85	34.5	32.7	11.6	21.2

The analysis of CD spectra of immobilized BLG-B demonstrated almost 7% and 5% loss of α-helix and β-sheet in the range of 25 to 85 °C, respectively (Table 1b). These results confirmed that BLG-B complex with [n-$PrNH_2$-KIT-6] has greater structural stability due to the interactions of BLG-B with the mesoporous surfaces, and the conformation of BLG-B conserves β-strand structure. In summary, increased resistance to heat denaturation induced by new and additional linkages between protein and mesoporous surfaces was observed.

Measurements of protein tertiary structure can be obtained by solvent accessibility assays of active chromophores in proteins such as aromatic amino acid residues. Protein denaturation, where the protected hydrophobic core of a globular protein becomes exposed to the solvent, can be monitored by changes in the near-UV CD spectrum in the region of 260–340 nm.

The near-UV CD spectrum of the BLG-B (Fig. 3 (a, b)) has negative peaks between 290 and 310 nm, resulting from two tryptophans (Trp19 and 61), four tyrosines (Tyr 20, 42, 99, and 102), and four phenylalanines (Phe 82, 105, 136, and 151). All of these amino acids may contribute to the CD signal. However, Trp residues are the main contributors to the near-UV CD signal.

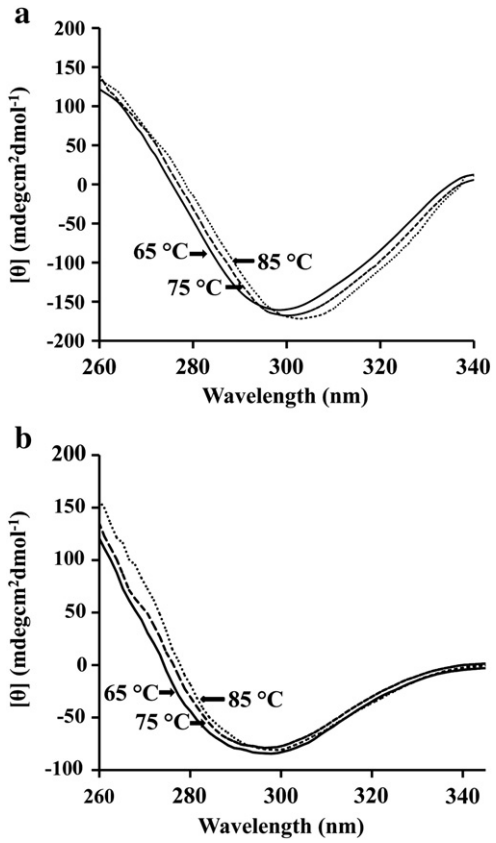


Fig. 3. Near-UV CD spectra of free (a) and immobilized BLG-B (b) measured at different temperatures of 65, 75 and 85 °C in 20 mM phosphate buffer, pH = 7.8.

Furthermore, Tyr and Phe generally do not absorb above 270 and 290 nm, respectively. Therefore, the negative bands above 290 nm can be attributed to asymmetrically perturbed Trp residues [50–53].

Thermally-induced structural changes were studied by near-UV CD spectroscopy of solutions of the free and immobilized BLG-B after heating the samples for 15 min at 65 °C, 75 °C and 85 °C. Increasing temperature from 65 to 85 °C induced a significant and measurable red shift in the ellipticity (6 nm) of free BLG-B (Fig. 3 (a)) whereas no detectable changes were monitored for ellipticities in the near-UV CD region in the case of immobilized BLG-B (Fig. 3 (b)). In light of accessibility data, a red-shifted ellipticity at 299 nm upon thermal stress is attributable to more exposure of the Trp component in free BLG-B in comparison with the immobilized BLG-B. When BLG is immobilized on the [n-$PrNH_2$-KIT-6] nanoparticles the partial restoration of ellipticities at 299 nm is observed.

It was suggested that temperature-induced unfolding of BLG-B disrupts the anisotropic environment of Trp residues of free BLG relative to immobilized BLG-B. In other words, this indicates that for free BLG-B thermal unfolding of the whole molecule over the temperature range measured results in increased access of the solvent to the tryptophan residues.

Further argument supporting the increase of heat stability of BLG-B after immobilization on the [n-$PrNH_2$-KIT-6] was supplied by BLG-B melting experiments. We used far-UV CD spectroscopy to determine the melting temperatures (Tm) of free and immobilized BLG-B. The values of Tm of free and immobilized BLG-B were determined, respectively, by monitoring their [$\theta_{218\ nm}$] values as a function of temperature from 40 to 100 °C with a rate of 1 °C/min (Fig. 4). For each monitored transition, the Tm of tested solution was determined as the transition midpoint of the melting curve. It can be seen from Fig. 4 that the Tm of free BLG-B is 70.2 ± 1 °C, whereas, the observed Tm of immobilized one is 89.5 ± 1 °C.

The significant increase in Tm (ΔT = 19 °C) in immobilized BLG-B measured by CD is in agreement with our previous observations on the great efficiency of immobilization for increased structural heat stabilities of the protein. It is also noteworthy to mention that immobilization provided not only partial protection against the secondary structural changes as temperature increases, but also renders more stable tertiary structure. It is widely documented that any increase in Tm should result in an increase in protein stability. Therefore, an increase in Tm may indicate that immobilization has a stabilizing effect on protein structure.

A possible reason for the increase in the Tm in the immobilized protein may be the stabilization of several loops, which may become more firmly held upon immobilization and subsequent confinement. Confinement effect provides a more rigid external backbone for BLG-B molecules, where as the effect of higher temperatures in

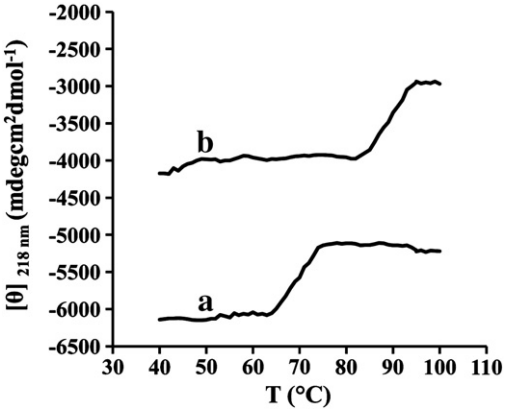


Fig. 4. Temperature-induced melting curves of free BLG-B (a) and immobilized BLG-B (b) at pH 7.8, 20 mM phosphate buffer, determined by recording the molar ellipticity changes at 218 nm.

breaking the new and additional linkages that were established upon immobilization, became less prominent, thus increasing the thermal stability of the immobilized BLG-B. The increased thermal denaturation temperature (Tm) of BLG-B upon immobilization is also in a good agreement with observed increase of thermal aggregation temperature (Ta).

After Ta and Tm measurements (30 min), leaching test was applied to the samples to get clear stability information upon immobilization. We found the small amount of free BLG-B (13%) in the supernatant that is not large enough to interfere with our aforementioned measurements. However, it seems likely that in the presence of temperature, the unfolding process will postpone after the immobilization of BLG-B onto the nanoporous support.

3.4. Fluorescence studies

Fluorescence spectroscopy is a useful technique for studying the structure of protein molecules in solution. The intrinsic fluorescence of tryptophanyl residues is widely used in protein structural studies. In particular, the phenomenon of fluorescence quenching has great application since it is suggested to reflect the accessibility of apolar amino acids, i.e. the fluorescent residues, to the quenching molecule [54]. This technique has been applied mainly to soluble proteins. However, it is potentially interesting also for immobilized proteins, and it has indeed been successfully used for this purpose in previous studies [39,55–58]. One of the most well known water-soluble quenchers is acrylamide [59].

Bovine BLG-B has two Trp residues: Trp-19 and Trp-61. From crystal structure studies [47], Trp19 is placed at the base of the central hydrophobic calyx of the protein, while Trp 61 is located on the external loop. Therefore, the intrinsic fluorescence of BLG-B is mostly attributed to Trp19, positioned in a more apolar environment than Trp 61 [60].

Acrylamide was used as a fluorescence quenching probe to investigate exposure of Trp residues on the protein surface for free and immobilized BLG-B upon heat treatment. Tryptophan quenching of free and immobilized BLG-B reveals their interaction with acrylamide. The fluorescence quenching data were analyzed by the Stern–Volmer equation, Eq. 1 [61]:

$$F_0/F = 1 + Ksv[Q] \quad (1)$$

where F_0 and F are the steady-state fluorescence intensities in the absence or presence of quencher, respectively, Ksv is the Stern–Volmer quenching constant, and [Q] is the concentration of quencher. The values of Ksv at different temperatures are shown in Table 2. The linearity of the F_0/F versus [Q] plot is shown in Fig. 5. As shown in Table 2, the quenching constant Ksv increases with increasing temperature, indicating that the probable quenching mechanism of BLG-B is a dynamic quenching procedure involving complexation between acrylamide and BLG-B.

The dependence of quenching on acrylamide concentration is linear. Linearity in the Stern–Volmer plot excludes the occurrence of protein denaturation, because this would produce an upward curving plot. The slope of immobilized BLG-B (Fig. 5 (b)) is lower than that of the free BLG-B (Fig. 5 (a)) at all temperatures studied (65–75 and 85 °C),

Table 2
Acrylamide quenching of free and immobilized BLG-B as a function of temperature. The values of Ksv (M^{-1}) were determined for each temperature as described in Materials and methods and represent the typical average of three measurements.

Temperature (°C)	Ksv (M^{-1}) for BLG-B	Ksv (M^{-1}) for immobilized BLG-B
65	3.2	2.4
75	3.6	2.7
85	5.1	3.6

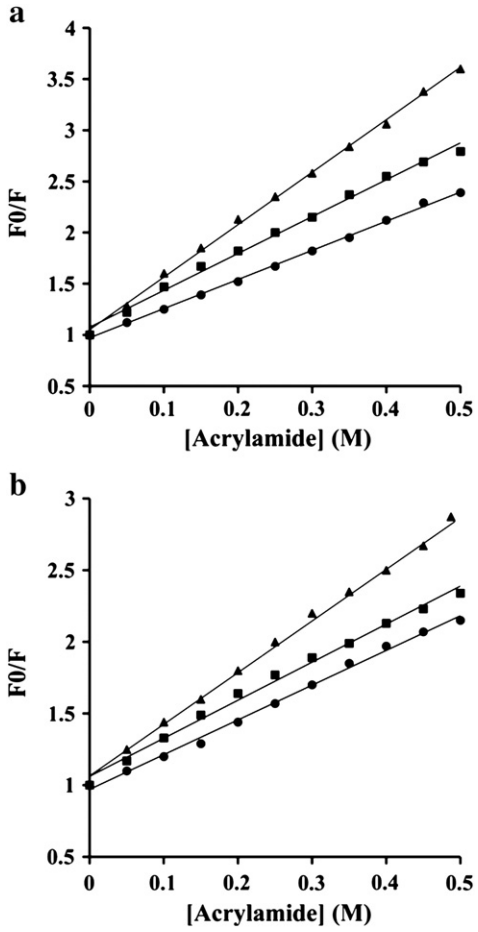


Fig. 5. Representative Stern–Volmer plot analysis of the acrylamide quenching of free (a) and immobilized BLG-B (b) At 65 (circles), 75 (squares) and 85 °C (triangles).

suggesting that an appreciable tightening of the structure around tryptophans and/or a further shielding of these residues from collisions occurs upon immobilization.

The Ksv values at 65 °C for immobilized and free BLG-B are 2.4 and 2.8 M^{-1}, respectively. More importantly, the Ksv increment is higher for free BLG-B (by 1.92 times) than immobilized BLG-B (1.50 times) in the range of 65–85 °C (Table 2). Overall, these results suggest that the accessibility of acrylamide to fluorophores is higher in the free BLG-B than in the immobilized-BLG-B.

The structural stabilities of BLG-B induced by its immobilization on amine-functionalized mesoporous materials require more discussion. It is amply documented that the main driving force in stabilization of proteins upon their immobilization is due to establishing new interactions between proteins and mesoporous walls [46,47,62]. Additionally, upon immobilization the apparent volume of the protein environment decreases and the protein becomes denser acquiring more stable conformational structure. This property of the mesoporous materials has been correlated with its ability to protect the structures and functions of proteins against heat inactivation. Since polar groups are present on the surface of mesoporous materials as well as the protein, their hydration is favorable for hydrogen bond formation.

It is well documented that proteins are more hydrated inside silica pores. As a consequence, it is also expected to help increase protein thermal stability. It is believed that Hofmeister ions, and hence also the presence of silanol groups on the silica surface, might influence the embedded protein structure indirectly through changes in the physical and hydrogen-bonding properties of water, which might be the reason for increased hydration of the protein. Part of this effect can also be attributed to a decrease of the rotational and translational

dynamics of the system [63]. In addition to confinement effects and establishing hydrogen bonds, electrostatic interactions also play a key role in increasing the stability of immobilized proteins. This finding is of particular interest in potential uses of BLG as a vector for pharmaceuticals into the gut, since it would protect BLG and its complexes with pharmacophores of interest in the highly hydrolytic surroundings of the GIT (gastrointestinal tract). This could enable entirely novel uses of this highly abundant protein, which can be produced on an industrial scale for human and veterinary pharmaceutical supplies. The synthesis of previously unknown derivatives of mesoporous silica nanoparticles able to bind proteins inside their pores also creates large opportunities to form stabilized enzymes with significantly increased heat stability, because they are sheltered against proteolytic and hydrolytic deactivation when hidden and stabilized inside the pores of the silica. Enzymes or vectors such as BLG could in this way be given properties approaching those of naturally occurring heat- and/or pH-tolerant extremophilic enzymes, and would likely be rendered resistant to an even more diverse set of elements than natural enzymes, which are usually stabilized against either heat or pH or molecular strength only.

4. Conclusions

In summary, we have investigated the ability of [n-PrNH_2-KIT-6] for application in the immobilization of BLG-B. It was shown spectroscopically that [n-PrNH_2-KIT-6] has a great potential for adsorption and retention of BLG-B. UV visible studies showed that immobilization prevents the thermally induced aggregation of BLG-B. Far and near-UV CD demonstrated that the secondary and tertiary structures of immobilized BLG-B were more stable against temperature in comparison with free BLG. Measurement of the melting temperature showed that the Tm of immobilized BLG was 19 °C higher than that of free BLG. Acrylamide fluorescence quenching studies of free and immobilized BLG-B showed that immobilized protein was less prone to Trp-residue quenching due to increased structural stability that has occurred owing to new interactions between protein and the mesoporous silica surfaces. In the result a very promising new material was produced which may greatly improve the formulation of pharmaceuticals sensitive to different degradations during administration and which may also allow to protect several industrial enzymes exposed otherwise when in free form, to different forms the denaturation.

Acknowledgment

The financial support of the Iran National Science Foundation (INSF) and the support of the Research Council of the University of Tehran are gratefully acknowledged. We are grateful to Tyler Arbour (Ph.D. student in Berkeley University of USA) for his support in editing the English language of our paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2012.02.006.

References

[1] J.S. Beck, J.C. Vartuli, W.J. Roth, M.E. Leonowicz, C.T. Kresge, K.D. Schmitt, A new family of mesoporous molecular sieves prepared with liquid crystal templates, Journal of the American Chemical Society 114 (1992) 10834–10843.
[2] J.F. Diaz, K.J. Balkus, Enzyme immobilization in MCM-4 1 molecular sieve, Journal of Molecular Catalysis B: Enzymatic 2 (1996) 115–126.
[3] J. Deere, E. Magner, J.C. Wall, B.K. Hodnett, Oxidation of ABTS by silicate-immobilized cytochrome c in nonaqueous solutions, Biotechnology Progress 19 (2003) 1238–1243.
[4] D. Goradia, J. Cooney, K. Hodnett, E. Magner, Characteristics of a mesoporous silicate immobilized trypsin bioreactor in organic media, Biotechnology Progress 22 (2006) 1125–1131.
[5] E.L. Pires, E.A. Mira, G.P. Valenca, Gas-phase enzymatic esterification on immobilized lipases in MCM-41 molecular sieves, Applied Biochemistry and Biotechnology 98 (2002) 963–976.
[6] A. Salis, D. Meloni, S. Ligas, M.F. Casula, M. Monduzzi, V. Solinas, Physical, chemical adsorption of mucor javanicus lipase on SBA-15 mesoporous silica synthesis, structural characterization, activity performance, Langmuir 21 (2005) 5511–5516.
[7] E. Serra, A. Mayoral, Y. Sakamoto, R.M. Blanco, I. Diaz, Immobilization of lipase in ordered mesoporous materials, effect of textural and structural parameters, Microporous and Mesoporous Materials 114 (2008) 201–213.
[8] N.W. Fadnavis, V. Bhaskar, M.L. Kantam, B.M. Choudary, Highly efficient "Tight Fit" immobilization of α-chymotrypsin in mesoporous MCM-41: a novel approach using precursor immobilization and activation, Biotechechnology Progress 19 (2003) 346–351.
[9] Y. Lu, Y. Guo, Y. Wang, X. Liu, Y. Wang, Y. Guo, Immobilized penicillin G acylase on mesoporous silica, the influence of pore size, pore volume, mesophases, Microporous and Mesoporous Materials 114 (2008) 507–510.
[10] A. Vinu, N. Gokulakrishnan, V.V. Balasubramanian, S. Alam, M.P. Kapoor, K. Ariga, Three-dimensional ultralarge-pore Ia3d mesoporous silica with various pore diameters, their application in biomolecule immobilization, Chemistry A European Journal 14 (2008) 11529–11538.
[11] K. Hisamatsu, T. Shiomi, S.I. Matsuura, Y.N. Takayuki, T. Tsunoda, F. Mizukami, α-Amylase immobilization capacities of mesoporous silicas with different morphologies, surface properties, Journal of Porous Materials 1 (2011) 1–8.
[12] F. Kleitz, S.H. Choi, R. Ryoo, Cubic Ia3d large mesoporous silica, synthesis, replication to platinum nanowires, carbon nanorods, carbon nanotubes, Chemical Communications 17 (2003) 2136–2137.
[13] B. Menaa, M. Herrero, V. Rives, M. Lavrenko, D.K. Eggers, Favourable influence of hydrophobic surfaces on protein structure in porous organically-modified silica glasses, Biomaterials 29 (2008) 2710–2718.
[14] G. Kontopidis, C. Holt, L. Sawyer, Beta-lactoglobulin: binding properties, structure, and function, Journal of Dairy Science 87 (2004) 785–796.
[15] L.K. Creamer, D.A.D. Parry, G.N. Malcom, Secondary structure of bovine b-lactoglobulin B, Archives of Biochemistry and Biophysics 227 (1983) 98–105.
[16] M.A. Hoffman, G. Sala, C. Olieman, G.C. De Kruif, Molecular mass distributions of heatinduced beta-lactoglobulin, Journal of Agricultural and Food Chemistry 45 (1997) 2949–2957.
[17] S. Cairoli, S. Iametti, F. Bonomi, Reversible and irreversible modifications of betalactoglobulin upon exposure to heat, Journal of Protein Chemistry 13 (1994) 347–354.
[18] E. Dufour, T. Haertle, Temperature-induced folding changes of beta-lactoglobulin in hydro-methanolic solutions, International Journal of Biological Macromolecules 15 (1993) 293–297.
[19] P. Relkin, Reversibility of heat-induced conformational changes, surface exposed hydrophobic clusters of beta-lactoglobulin, their role in heat-induced sol–gel state transition, International Journal of Biological Macromolecules 22 (1998) 59–66.
[20] R.K.O. Apenten, S. Khokhar, D. Galani, Stability parameters for beta-lactoglobulin thermal dissociation, unfolding in phosphate buffer at pH 7.0, Food Hydrocolloids 16 (2002) 95–103.
[21] D. Galani, R.K.O. Apenten, Revised equilibrium thermodynamic parameters for thermal denaturation of β-lactoglobulin at pH 2.6, Thermochimica Acta 363 (2000) 137–142.
[22] Y.D. Livney, D.G. Dalgleish, Specificity of disulfide bond formation during thermal aggregation in solutions of beta-lactoglobulin B and k-casein A, Journal of Agricultural and Food Chemistry 52 (2004) 5527–5532.
[23] N. Poklar, G. Vesnaver, S. Lapanje, Studies by UV spectroscopy of thermal denaturation of β-lactoglobulin in urea, alkylurea solutions, Biophysical Chemistry 47 (1993) 143–151.
[24] Y. Fang, D.G. Dalgleish, Conformation of β-lactoglobulin studied by FTIR, effect of pH, temperature, adsorption to the oil–water interface, Journal of Colloid and Interface Science 196 (1997) 292–298.
[25] X.L. Qi, C. Holt, D. Mcnulty, D.T. Clarke, S. Brownlow, G.R. Jones, Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD, IR spectroscopy, a test of the molten globule hypothesis, The Biochemical Journal 324 (1997) 341–346.
[26] D. Galani, R.K.O. Apenten, Heat induced denaturation and aggregation of betalactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregation, International Journal of Food Science and Technology 34 (1994) 467–476.
[27] L. Mortier, A. Braekman, D. Cartuyvels, R. Van Renterghem, J. De Block, Intrinsic indicators for monitoring heat damage of consumption milk, Biotechnologie, Agronomie, Société et Environnement 4 (2004) 221–225.
[28] M. Villamiel, F. Lopez, R. Ino, N. Corzo, A. Olano, Denaturation of β-lactoglobulin, native enzymes in the plate exchanger, holding tube section during continuous flow pasteurization of milk, Food Chemistry 58 (1997) 49–52.
[29] L. Claeys, A. Van Loey, M. Hendrickx, Intrinsic time temperature integrators for heat treatment of milk, Trends in Food Science and Technology 13 (2002) 293–311.
[30] G.H. Mcintosh, G.D. Regester, R.K. Lelue, P.J. Royle, G.W. Smithers, Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats, The Journal of Nutrition 125 (1995) 809–816.
[31] A. Divsalar, A.A. Saboury, H. Mansoori-Torshizi, F. Ahmad, Design, synthesis, biological evaluation of a new palladium (II) complex, beta-lactoglobulin, K562 as targets, The Journal of Physical Chemistry B 114 (2010) 3639–3647.
[32] D.R. Radu, C.Y. Lai, K. Jeftinija, E.W. Rowe, S. Jeftinija, V.S.Y. Lin, A polyamidoamine dendrimer-capped mesoporous silica nanosphere-based gene transfection reagent, Journal of the American Chemical Society 126 (2004) 13216–13217.

[33] D.M. Huang, Y. Hung, B.S. Ko, S.C. Hsu, W.H. Chen, C.L. Chien, C.P. Tsai, Y.C. Chen, Highly efficient cellular labeling of mesoporous nanoparticles in human mesenchymal stem cells, Implication for stem cell tracking, The FASEB Journal 19 (2005) 2014–2016.
[34] Y.S. Lin, C.P. Tsai, H.Y. Huang, C.T. Kuo, Y. Hung, D.M. Huang, Y.C. Chen, C.Y. Mou, Well-ordered mesoporous silica nanoparticles as cell markers, Chemistry of Materials 17 (2005) 4570–4573.
[35] I. Slowing, B.G. Trewyn, V.S.Y. Lin, Effect of surface functionalization of MCM-41-type mesoporous silica nanoparticles on the endocytosis by human cancer cells, Journal of the American Chemical Society 128 (2006) 14792–14793.
[36] S. Radin, G. El-Bassyouni, E.J. Vresilovic, E. Schepers, P. Ducheyne, In vivo tissue response to resorbable silica xerogels as controlled-release materials, Biomaterials 26 (2005) 1043–1052.
[37] T.H. Chung, S.H. Wu, M. Yao, C.W. Lu, Y.S. Lin, Y. Hung, The effect of surface charge on the uptake and biological function of mesoporous silica nanoparticles 3T3-L1 cells and human mesenchymal stem cells, Biomaterials 28 (2007) 2959–2966.
[38] C. Moitzi, L. Donato, C. Schmitt, L. Bovetto, G. Gillies, A. Stradner, Structure of b-lactoglobulin microgels formed during heating as revealed by small-angle X-ray scattering and light scattering, Food Hydrocolloids 25 (2011) 1766–1774.
[39] M. Falahati, L. Ma'mani, A.A. Saboury, A. Shafiee, A. Foroumadi, A.R. Badiei, Aminopropyl-functionalized cubic Ia3d mesoporous silica nanoparticle as an efficient support for immobilization of superoxide dismutase, Biochimica et Biophysica Acta 1814 (2011) 1195–1202.
[40] G.B. Merlini, A.A. Vittorio, G. Palladini, L. Obici, S. Casarini, V. Perfetti, Protein aggregation, Clinical Chemistry and Laboratory Medicine 39 (2001) 1065–1075.
[41] L.L. Iversen, S.R.J. Mortishire, S.J. Pollack, M.S. Shearman, The toxicity in vitro of b-amyloid protein, The Biochemical Journal 311 (1995) 1–16.
[42] D.S. Maclean, Q. Qian, C.R. Middaugh, Stabilization of proteins by low molecular weight multi-ions, Journal of Pharmaceutical Sciences 91 (2002) 2220–2230.
[43] T.P. Primm, K.W. Walker, H.F. Gilbert, Facilitated protein aggregation, The Journal of Biological Chemistry 271 (1996) 33664–33669.
[44] K. Wang, B. Kurganov, Kinetics of heat-and acidification-induced aggregation of firefly luciferase, Biophysical Chemistry 106 (2003) 97–109.
[45] L. Ronda, S. Bruno, C. Viappiani, S. Abbruzzetti, A. Mozzarelli, K.C. Lowe, S. Bettati, Circular dichroism spectroscopy of tertiary and quaternary conformations of human hemoglobin entrapped in wet silica gels, Protein Science 15 (2006) 1961–1967.
[46] I. Tetsuji, I. Ryo, E. Takeo, H. Takaaki, I. Takuji, U. Yoko, F. Yoshiaki, M. Fujio, Effective immobilization of subunit protein in mesoporous silica modified with ethanol, Biotechnology and bioengineering 97 (2007) 200–205.
[47] B. Chen, C. Lei, Y. Shin, J. Liu, Probing mechanisms for enzymatic activity enhancement of organophosphorus hydrolase in functionalized mesoporous silica, Biochemical and Biophysical Research Communications 4 (2009) 1177–1181.
[48] B.Y. Qin, M.C. Bewley, L.K. Creamer, E.N. Baker, G.B. Jameson, Functional implications of structural differences between variants A, B of bovine beta-lactoglobulin, Protein Science 8 (1999) 75–83.
[49] S.P.F.M. Roefs, C.G. De Kruif, A model for the denaturation, aggregation of β-lactoglobulin, European Journal of Biochemistry 226 (1994) 883–889.
[50] Y.Q. Wang, H.M. Zhang, G.C. Zhang, W.H. Tao, S.H. Tang, Binding of brucine to human serum albumin, Journal of Molecular Structure 830 (2007) 40–45.
[51] M.I. Viseu, T.I. Carvalho, S.M.B. Costa, Conformational transitions in β-lactoglobulin induced by cationic amphiphiles, equilibrium studies, Biophysical Journal 86 (2004) 2392–2402.
[52] F. Zsila, T. Imre, P.T. Szabó, Z. Bikádi, M. Simonyi, Induced chirality upon binding of cis-parinaric acid to bovine beta-lactoglobulin, spectroscopic characterization of the complex, FEBS Letters 520 (2002) 81–87.
[53] A. Divsalar, A.A. Saboury, A.A. Moosavi- Movahedi, Conformational structural analysis of β-lactoglobulin-A upon interaction with Cr^{+3}, The Protein Journal 25 (2006) 157–165.
[54] M.R. Eftink, C.A. Ghiron, Fluorescence quenching studies with proteins, Analytical Biochemistry 114 (1981) 199–227.
[55] J.K. Raynes, F.G. Pearce, S.J. Meade, J.A. Gerrard, Immobilization of organophosphate hydrolase on an amyloid fibril nanoscaffold. Towards bioremediation and chemical detoxification, Biotechnology Progress 27 (2011) 360–367.
[56] J.H. Kleinschmidt, P.V. Bulieris, J. Qu, M. Dogterom, T. Den Blaauwen, Association of neighboring β-strands of outer membrane protein A in lipid bilayers revealed by site-directed fluorescence quenching, Journal of Molecular Biology 407 (2011) 316–332.
[57] A. Kyrychenko, I.Y. Sevriukov, Z.A. Syzova, A.S. Ladokhin, A.O. Doroshenko, Partitioning of 2, 6-Bis (1H-Benzimidazol-2-yl) pyridine fluorophore into a phospholipid bilayer, complementary use of fluorescence quenching studies, molecular dynamics simulations, Biophysical Chemistry 154 (2011) 8–17.
[58] A. Pundle, A. Prabhune, M. Sastry, Invertase-lipid biocomposite films: preparation, characterization, and enzymatic activity, Biotechnology Progress 20 (2004) 155–161.
[59] M.R. Eftink, C.A. Ghiron, Does the fluorescence quencher acrylamide bind to proteins? Biochimica et Biophysica Acta 916 (1987) 343–349.
[60] C.A.M. Portugal, J.G. Crespo, J.C. Lima, Anomalous "unquenching" of the fluorescence decay times of β-lactoglobulin induced by the known quencher acrylamide, Journal of Photochemistry and Photobiology B, Biology 82 (2006) 117–126.
[61] A.B. Naik, L.R. Naik, J.S. Kadadevarmath, H. Pal, V.J. Rao, Fluorescence quenching of anthrylvinyl acetate by carbon tetrachloride, Journal of Photochemistry and Photobiology A: Chemistry 214 (2010) 145–151.
[62] J. Lei, J. Fan, C. Yu, L. Zhang, S. Jiang, B. Tu, D. Zhao, Immobilization of enzymes in mesoporous materials: controlling the entrance to nanospace, Microporous and Mesoporous Materials 73 (2004) 121–128.
[63] R. Ravindra, S. Zhao, H. Gies, R. Winter, Protein encapsulation in mesoporous silicate, the effects of confinement on protein stability, hydration, volumetric properties, Journal of the American Chemical Society 126 (2004) 12224–12225.