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Chitosan–bovine serum albumin complex formation: A model to design an enzyme isolation method by polyelectrolyte precipitation

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ABSTRACT

Interactions between a model protein (bovine serum albumin—BSA) and the cationic polyelectrolyte, chitosan (Chi), have been characterized by turbidimetry, circular dichroism and fluorescence spectroscopy. It has been found that the conformation of the BSA does not change significantly during the chain interaction between BSA and chitosan forming the non-covalently linked complex. The effects of pH, ionic strength and anions which modify the water structure around BSA were evaluated in the chitosan–BSA complex formation. A net coulombic interaction force between BSA and Chi was found as the insoluble complex formation decreased after the addition of NaCl. Around 80% of the BSA in solution precipitates with the Chi addition. A concentration of 0.05% (w/v) Chi was necessary to precipitate the protein, with a stoichiometry of 6.9 g BSA/g Chi. No modification of the tertiary and secondary structure of BSA was observed when the precipitate was dissolved by changing the pH of the medium. Chitosan proved to be a useful framework to isolate proteins with a slightly acid isoelectrical pH by means of precipitation.

1. Introduction

Chitosan (Chi) is a cationic polymer, which is derived from chitin, an abundant polymer in nature [1,2]. Chitin is the primary structural component of the outer skeletons of crustaceans and it is also found in many other species such as mollusks, insects and fungi. Chi is commonly obtained from crustacean chitin taken from crab and shrimp shell wastes. This polymer has a wide variety of commercial and biomedical applications that are related to its physical properties [2–6].

Chi in solution, as a weak polybase, exhibits a pH-dependent behaviour owing to the large quantities of amino groups on its chain. The mechanism by which chitosan is soluble in aqueous medium involves the protonation of amine groups of the polymer under low pH conditions. This protonation leads to chain repulsion, diffusion of protons, counter ions and water into the Chi and dissociation of secondary interactions. Owing to its positively charged amine groups, Chi interacts with negatively charged molecules through coulombic forces [6]. If the negatively charged molecules are proteins that are in a complex mixture, non-soluble complexes can be formed and then separated from the other macromolecules by precipitation.

Different biotechnological processes consume high amounts of enzymes, therefore, the production of enzymes is a pursuit central to the modern biotechnology industry. The obtaining of an enzyme is achieved from its natural source or by a genetically modified microorganism; this is known as upstream processing. The following process is called the downstream processing. This term is used to point out everything that comes after fermentation and consists of isolation and purification of the enzyme. This latter process has become important because it often represents up to 80% of the total cost of the production process of an enzyme [7,8]. Most purification technologies use precipitation of proteins as one of the initial operations aimed at concentrating the product for further downstream steps [9–11].

Polyelectrolyte precipitation uses a poly-charged polymer with opposite electrical charge to the target protein. Both, polyelectrolyte and protein, form non-covalent protein–polyelectrolyte soluble complexes under certain experimental conditions, and these complexes can interact with each other producing insoluble macro-aggregates [12,13]. This is a suitable method for protein isolation due to the very low polyelectrolyte concentrations used (up to 0.1%, w/v). This method offers the possibility of concentrating and purifying the target protein at low cost and the insoluble complexes can be re-dissolved by a change in pH or in the ionic strength to recover the target protein [14,15].

Abbreviations: BSA, bovine serum albumin; Chi, chitosan.

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In this work, we have studied the capacity of chitosan and a model protein such as bovine serum albumin (pI ≈ 5) to form complexes with the aim of applying this protein precipitation technique to the isolation of enzymes on a large-scale.

2. Materials and methods

2.1. Chemical

Bovine serum albumin (BSA), and chitosan (Chi) – minimum 75% deacetylation grade, given by the manufacturer – were purchased from Sigma Chem. Co. and used without further purification. Chi was dissolved in acetic acid 0.1 M at a concentration of 2% (w/v). All other reagents were of analytical grade.

2.2. Solubility curves of Chi vs. the medium pH in the BSA presence

It is well known that the solubility of Chi is highly dependent on the pH of the medium. Solubility titration curves of Chi solutions were obtained to characterize the Chi used in this work and expressed as the medium absorbance at 420 nm vs. pH. The variation of the pH was obtained by adding NaOH or HCl aliquots, allowing the system to reach equilibrium; and then the pH and the Abs at 420 nm were measured. This was also carried out in the presence of BSA to test any possible BSA effect on the solubility behaviour of the Chi.

2.3. Albumin turbidimetric titration with chitosan

To assay the capacity of Chi to interact with the BSA, we have chosen a pH value of 6.0 to carry out the Chi–BSA complex formation. This value was found not only to be the pH at which the Chi is soluble but also at which the BSA–chitosan mixture became non-soluble. Under this pH value, BSA has a net negative electrical charge value around −8 [16]. The formation of the insoluble non-covalent linked Chi–BSA complexes was monitored by turbidimetric titration [12,14]. Tris–HCl, sodium citrate buffer solutions with a fixed BSA concentration were titrated at 25 °C in a glass cell with the Chi solution as the titrant. To avoid changes in pH during titration, both the BSA and the Chi solutions were adjusted to the same pH value. The absorbance at 420 nm of solution was used to follow the Chi–BSA complex formation and it was plotted vs. the total concentration of polymer in the test tube. These plots were fitted with the following hyperbolic equation:

$$\tau = \frac{\tau_{max}[\text{Chi}]}{[\text{Chi}]_{0.5} + [\text{Chi}]} \quad (1)$$

where τ and τ_{max} are the absorbance and the maximum absorbance of the solution respectively and $[\text{Chi}]_{0.5}$ is the Chi concentration at half of the maximum turbidity. This value was used to estimate the BSA/Chi mass ratio in the precipitated form of the complex:

$$\text{BSA/Chi mass ratio} = \frac{[\text{BSA}](\text{g/L})}{[\text{Chi}]_{0.5}(\text{g/L})} \quad (2)$$

Solution absorbancies were measured using a Jasco 520 spectrophotometer with a thermostatized cell of 1 cm of path length.

2.4. BSA thermal stability

Thermally induced unfolding was monitored by absorbance measurements at 280 nm, as it was reported previously [13]. Data was analyzed assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated and the absorptivity coefficients of both states were different. Non-linear least squares method was used to fit the absorbance vs. temperature (T) data. The temperature at the midpoint of denaturation (T_m) was determinated and the unfolded BSA fraction was calculated from:

$$\alpha = \frac{\text{Abs}_i - \text{Abs}_N}{\text{Abs}_D - \text{Abs}_N} \quad (3)$$

where α is the unfolded BSA fraction, Abs_N and Abs_D are the absorptivities of the native and unfolded states respectively, Abs_i is the absorbance at a given temperature. The equilibrium constant for the unfolded process can be calculated as follows:

$$K = \frac{\alpha}{1 - a} \quad (4)$$

the free energy change (ΔG°) was calculated as:

$$\Delta G^\circ = -RT \ln K \quad (5)$$

From a plot of ΔG° vs. T, the unfolded ΔS° was calculated according to:

$$\Delta S^\circ = -\left(\frac{\partial \Delta G^\circ}{\partial T}\right) \quad (6)$$

The enthalpic change (ΔH°) was calculated from the equation [17,18]:

$$\Delta H^\circ = \Delta G^\circ + T\Delta S^\circ \quad (7)$$

Absorbance measurements were carried out on a Jasco 550 spectrophotometer. The temperature of the sample was controlled by a Peltier heater and measured with a thermocouple immersed in the cuvette. The heating rate was 1 °C/min. The data absorbancies vs. temperature values were collected by using the software provided by the manufacturer of the instrument.

2.5. Circular dichroims spectra

Circular dichroism (CD) scan of BSA with and without Chi was carried out using a Jasco spectropolarimeter, model J-8150. The ellipticity values [θ] were obtained in millidegrees (mdeg) directly from the instrument. The cell pathlength of 0.1 cm was used for the spectral range 200–250 nm. In all cases, five scans were made in the absence and presence of BSA.

2.6. Quenching of the BSA native fluorescence by acrylamide

Fluorescence quenching is the decrease in quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with the quencher molecule. The quenching of the fluorescence of the BSA tryptophan residues was carried out by titration with acrylamide in the presence and absence of Chi. The data was analyzed using the mathematical model for the sphere of action according to Lakowicz [19]:

$$\frac{F^\circ}{F} = 1 + K_D[Q]e^{[Q]\nu N/1000} \quad (8)$$

where F° and F are the BSA fluorescence emissions at 340 nm when the BSA was excited at 280 nm in the absence and presence of the acrylamide respectively, K_D is the Stern Volmer constant related to the lifetime of the fluorophore and the diffusion coefficient of the quencher, [Q] the quencher (acrylamide) concentration, N the Avogadroĭs number and ν the volume of the "sphere of action", i.e., the sphere within which the probability of immediate quenching is one, and whose radius is only slightly larger than the addition of the radiuses of the fluorophore and quencher.

2.7. Measurements of the BSA concentration

The binding of green bromocresol to albumin at pH 4.2 was used to form a complex that was measured spectrophotometrically at 628 nm [20].

3. Results

3.1. Solubility curves of Chi vs. the pH of the medium in the BSA presence

Fig. 1 shows titration curves obtained in the absence and presence of BSA. These solubility curves allowed us to find the exact pH value at which the complexes were formed and the Chi was soluble. A typical sigmoidal titration curve was obtained in both cases. We have found that the titration curve of Chi in the BSA presence could not overlap the curve in the absence of BSA. This finding suggested that the presence of BSA significantly modified the solubility behaviour of Chi. BSA has an isoelectric point around 5. At higher pH values, BSA carries a negative net charge and it would begin to interact with the Chi, positively charged, by electrostatic attraction. The complexes formed present larger values of absorbance than the values of absorbance of the solution of Chi.

3.2. Turbidimetric titration of BSA with Chi

Fig. 2 shows the absorbance dependence at 420 nm when BSA was added with increasing amounts of Chi solution. It presents a curve with a hyperbolic shape; whereas the BSA concentration in the supernatant appeared to decrease as the precipitate was formed. However, at high Chi concentrations, the BSA precipitation was not completed. The supernatant BSA concentration curves vs. Chi concentration curves shows a plateau which indicates a remaining of 20% of BSA.

The stoichiometry of the insoluble complexes formation was calculated from the non-linear fitting of the turbidimetric titration curve (Eqs. (1) and (2)). The calculated BSA/Chi ratio was 6.9 g ± 0.3 g BSA/g Chi. This ratio corresponds to the final state of the ionic complex in its non-soluble form. It is well demonstrated that the formation of this ionic complex is produced by two steps [12,21,22]: (a) the formation of a primary protein–polymer complex, which is soluble; (b) the interaction among the soluble complexes to form larger non-soluble aggregates, which are determinated by turbidimetric titration. The previous stoichiometry ratio corresponds to the formation of the already mentioned non-soluble aggregates. Therefore, that value is an average of the BSA/Chi mass ratio in the precipitate.

In general, the protein–polyelectrolyte ratio value is relevant because it allows calculating the minimal polymer amount to precipitate the target protein. The BSA/Chi ratio value obtained is consistent with the results reported for the precipitation of other proteins by polyelectrolytes [23].

The total Chi concentration required to precipitate the maximum amount of BSA is very low (in the order of 0.05–0.07%, w/v) if compared with the quantity of classical protein precipitants as inorganic cations and anions [10,24] Therefore, Chi precipitation appears to be an excellent method to precipitate proteins, owing to the fact that it is a non-expensive method and needs small amounts of Chi.

When designing scaling-up methods, it is important to precipitate proteins by using a polyelectrolyte. The target proteins are present in high volume of solution so small masses of these polyelectrolytes are necessary to precipitate the desired protein completely.

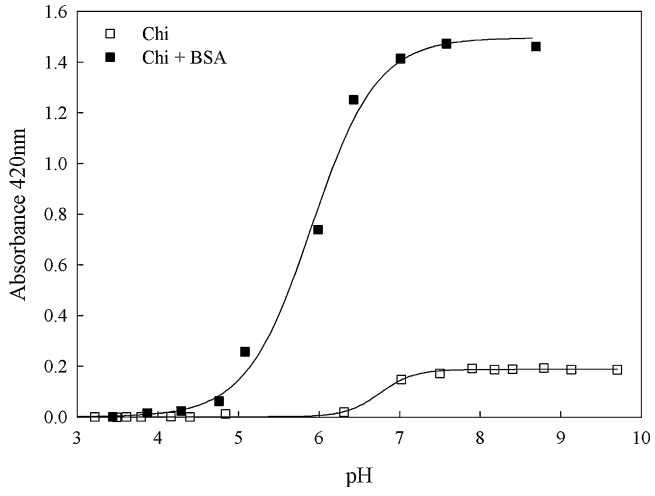

Fig. 1. Absorbance at 420 nm of Chi solution vs. the medium pH. Chi concentration 0.1% (w/v), the pH of the medium was varied by adding increasing amount of NaOH or HCl. The BSA concentration was 1 mg/mL. Temperature 25 °C. Medium acetate 50 mM–phosphate 50 mM.

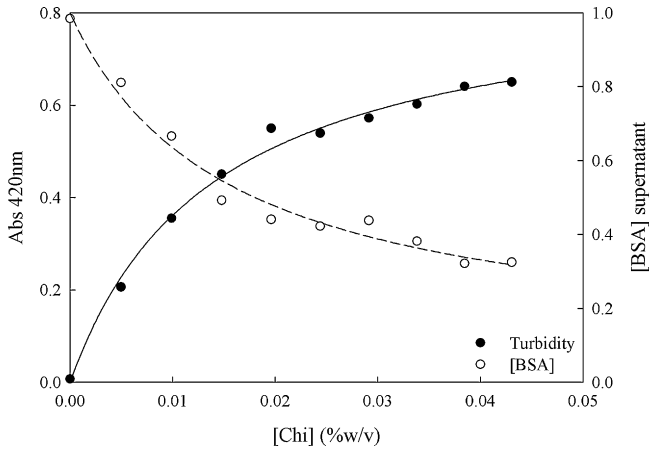

Fig. 2. Titration of BSA with increasing Chi concentration, medium buffer phosphate 50 mM, pH 6.0. Temperature 25 °C. The BSA concentration in the supernatant vs. Chi total concentration was also determined.

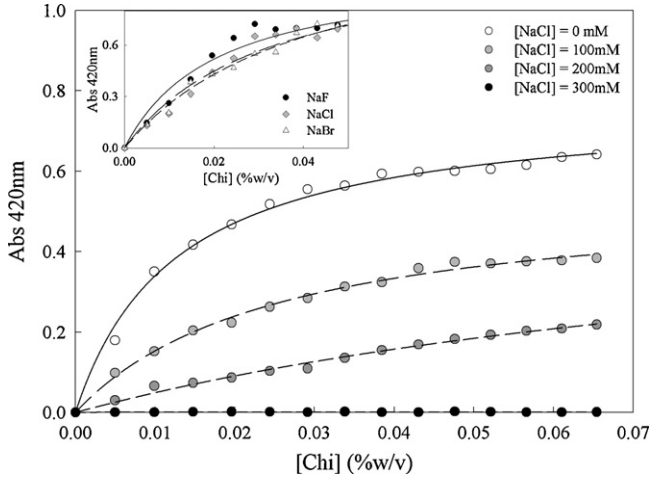

Fig. 3. Turbidimetric titration curve of BSA 1 mg/mL by Chi at increasing ionic strength given by NaCl. Medium condition: sodium phosphate buffer 50 mM, pH 6.0. Temperature 25 °C. Inset: turbidimetric titration curve of BSA 1 mg/mL by Chi in the presence of 100 mM of different sodium halides. Medium condition: sodium phosphate buffer 50 mM, pH 6.0. Temperature 25 °C.

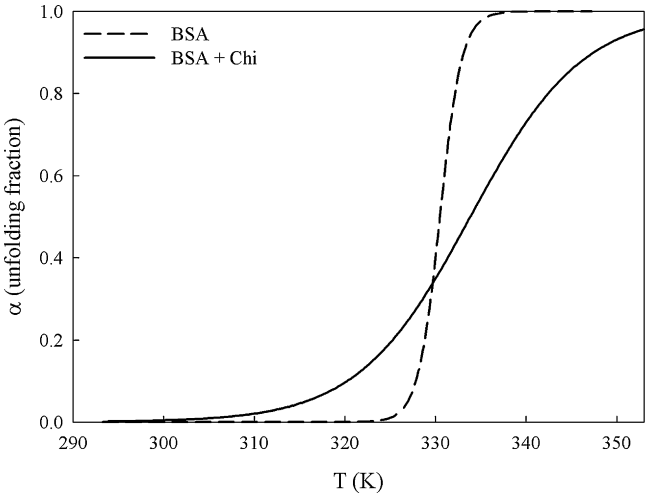

Fig. 4. Unfolding thermal shape of BSA 1 mg/mL in the absence and presence of Chi 0.1% (w/v), the data has been expressed as unfolding protein fraction vs. temperature. Heating rate: 1°/min. Medium buffer acetate/acetic acid 50 mM, pH 4.0.

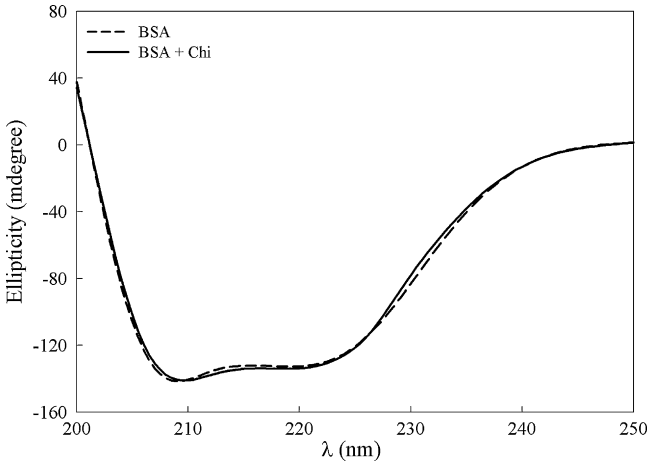

Fig. 5. CD spectra of BSA 1 mg/mL in the presence and absence of Chi 0.1% (w/v). Medium buffer acetate/acetic acid 50 mM, pH 4.0. Temperature 25 °C.

3.3. Turbidimetric titration of BSA with chitosan in presence of salts

The precipitation curves were carried out at different NaCl concentrations, as shown in Fig. 3. They were consistent with a net electrostatic mechanism proposed for this complex formation, since the BSA–Chi complexes dissolved gradually in the presence of NaCl. At a level of around 0.3 M, the BSA–Chi complex formation was completely inhibited.

The presence of sodium salt halides (Cl^-, Br^- and F^-) 100 mM was investigated on the complex formation. In the presence of these salts, superposed plots were obtained (see inset Fig. 3), suggesting that the ordered water molecules do not participate in the BSA–Chi interaction. Therefore, it appears that only the coulombic mechanism is the one which takes part in this interaction.

3.4. Thermal stability of BSA in the Chi presence

The interaction between a flexible chain polymer and a protein may induce conformational changes in the tertiary and secondary structure of the protein, with a loss in its thermodynamical stability and biological activity. The thermal unfolding of BSA was analyzed by measuring the protein absorbance change at 280 nm with increasing temperatures. Fig. 4 shows the dependence of the BSA unfolded fraction vs. the temperature change in the absence and presence of Chi. The presence of the polyelectrolyte changes the shape of the curve. We have found that there was a decrease in the middle zone of the curve, which was consistent with the loss of the cooperative effects associated with the unfolding process [18]. From these curves, the middle transition temperature (T_m) was calculated by a non-linear fitting of the data. Table 1 shows the values yielded; the thermodynamic function values are also included. The Chi presence induced a slightly thermodynamical stabilization of the BSA since an increase in the T_m value was observed.

It is well known that entropic and enthalpic changes are positive for the proteins thermal denaturation process. We have found the same behaviour for BSA. [18,25]. These positive changes were associated with the loss of ordered water molecules around the hydrophobic moieties of the BSA and with a change in its tertiary structure.

The presence of Chi, when interacting with the BSA, delivers the formation of a bigger and more stable structure. This fact is reflected in the increase of the T_m value and in the significant increase of the unfolding entropic change, which suggests that the Chi–BSA complex is more regular than the BSA alone in the initial state previous to the thermal unfolding. In addition, the increase of endothermicity in the BSA unfolding process in Chi presence is in agreement with a previous stabilization of the BSA initial state [26,27].

Fig. 4 also shows a significant change in the cooperativity of the unfolding process, the presence of Chi induced a decrease in the $(\partial\alpha/\partial T)$ value calculated at the T_m ($\alpha = 0.5$) in agreement with a loss of the BSA unfolding trend.

Table 1
Unfolding thermodynamic function values calculated from the thermal denaturation curves at 25 °C.

System	T_m (°C)	ΔS° (cal/mol K)	ΔH° (kcal/mol)	$(\partial\alpha/\partial T)$
BSA	57.4 ± 0.1	34 ± 1	20.1 ± 0.5	0.207 ± 0.009
BSA + Chi	60.7 ± 0.1	85 ± 2	28.7 ± 0.9	0.040 ± 0.002

3.5. Fluorescence and circular dichroism spectra of the BSA in the presence of the Chi

Spectroscopic techniques such as native fluorescence emission and circular dichroism are very useful to obtain information about the interaction between a protein and another molecule. Protein CD spectrum gives information about the changes induced in the secondary structure of the macromolecules. We have measured the circular dichroism spectrum of BSA in the absence and presence of Chi, and we have observed no significant modification in the CD spectrum, as shown in Fig. 5. This suggested that the Chi does not modify the secondary structure of the BSA. This finding is important because in the design process of a protein isolation and purification method, the polyelectrolyte used should not modify the secondary and tertiary structures of the target protein.

Chi did not induce any modifications in the native fluorescence spectrum of the protein (data not shown), which suggested that the Chi does not modify the environment of the tryptophan residues of the BSA. However, in order to obtain more information about the behaviour of the Chi interaction with the BSA surface, the quenching in the native fluorescence of tryptophan residues of BSA in the presence of Chi was assayed.

The native fluorescence quenching of a protein using a quencher, allowed us to obtain valuable information about the capacity of the quencher molecule to interact with the tryptophan residues accessible to the solvent. A wide variety of substances act as quenchers of fluorescence. Quenchers of indole, tryptophan, and its derivatives include acrylamide, succinimide and pyridinium [19].

Table 2
Values of K_D and volumes of the "sphere of action".

	K_D (L/mol)	v (nm^3)
BSA	3.75 ± 0.08	3.7 ± 0.3
BSA + Chi	3.8 ± 0.1	2.3 ± 0.2

In this work, BSA was titrated with acrylamide in the absence and presence of Chi; the data was expressed as a Stern Volmer plot ($F°/F$ vs. [Q]). Convex Stern Volmer plots were found for BSA (data not shown), which was consistent with a "sphere of action" mechanism. Applying Eq. (8), the quenching constant (K_D) and the sphere volume were calculated. The values are shown in Table 2. No difference in the K_D values was observed in the presence of Chi, which suggests no perturbation of the tryptophan residues of the BSA by the Chi. However, the volume of the sphere of action in the presence of Chi was diminished in agreement with a smaller volume available for the quencher around the tryptophan residues induced by the presence of Chi [19].

4. Discussion

Current methods of protein purification involve an extensive series of steps and processes that increase the cost of the final product. New techniques for large-scale protein separation are, therefore, of interest. One of these involves the addition of polyelectrolytes, leading to selective protein phase separation [14,15]. Proteins interact strongly with both synthetic and natural polyelectrolytes. These interactions are modulated by variables such as pH and ionic strength, and may result in the formation of soluble or insoluble complexes, or amorphous precipitates. This phase separation begins with the electrostatic interactions between the protein and the polyelectrolyte which, at very low ionic strength, results in tight ion-pairing.

There are a lot of synthetic polyelectrolytes proposed as protein precipitants; however, very few of them are non-toxic. This is important since most of the polyelectrolyte precipitates together with the target protein and cannot be removed. Chi is one of the few cationic biopolymers in nature. It is biocompatible, biodegradable, non-immunogenic, and non-toxic in animal tissues.

At present, the uses of Chi have been increased for the design of downstream processing methods in the biotechnological processes, owing to the fact that it is not expensive and has mild properties for the macromolecules.

Chi exhibits a pH-sensitive behaviour as a weak polybase owing to the large quantities of amino groups on its chain. The pK_a value of 7.00 corresponds to the pH value at mid-point of the Chi transition between the soluble and insoluble states and does not correspond to the mean pK_a of the amine groups of Chi which is determined by acid basic titration. However, both values are near because it is the deprotonation of the NH_2– which induces the flocculation of the Chi.

The native fluorescence quenching of a protein by a quencher is a way of allowing us to analyze the interaction between the tryptophan environment of a protein and the solvent. Thus, no Chi effect on the BSA surface was found, which suggests a poor interaction between Chi and the tryptophan environment. It has been reported that BSA interacts with various quencher by a sphere of action mechanism [19]. Chi did not modify the rate of the attack of the quencher (acrylamide) to the tryptophan (proved from the K_D value), however the sphere volume slightly decreased in the presence of Chi. This finding is a proof of the presence of Chi in the BSA domain, but its interaction with Chi is not strong enough to avoid the quencher's attack to the environment of the BSA tryptophan.

The difference in thermostability between BSA and BSA–Chi was found to be of around +3 °C, and this suggested that Chi increased the thermodynamical BSA stability. This finding was attributed to the presence of both or one of the following mechanisms: (1) the coulombic interaction between the negatively charge groups of BSA (carboxylic groups), and the amino groups of Chi, which lead to the formation of a more rigid and stable, thermodynamical structure and, (2) an increase in the BSA hydration due to the presence of the Chi chains near the BSA domain. This last process is in agreement with the decrease of the unfolding cooperative process, observed in the heating curve of the BSA in the presence of Chi.

One of the most important findings from the present results was that the Chi does not modify the secondary and tertiary structure of the BSA. This finding was determined by the CD spectrum of the dissolved BSA–Chi complexes and was consistent with the result from the fluorescence emission of the native BSA.

The polyelectrolyte precipitation uses very low polyelectrolyte concentration which makes this technique non-expensive. Chi allows us to precipitate BSA with a total polymer concentration of around 0.05% (w/v), which makes the uses of this polymer very interesting from the economical point of view. In addition, we have found that the solution exhibited low viscosity and, therefore, it was easy to scale. The use of a non-toxic and natural polymer such as Chi, adds another favourable quality to this methodology. However, it is necessary to develop experimental measurements to determine the influences of experimental variables of the medium on the complex formation and dissolution to apply this technique to the precipitation and isolation of other acidic proteins.

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