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Protein unfolding on interfaces: a structural and functional study

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“Je grimpe pour me sentir en harmonie avec moi-même, parce que je vis dans l’instant, parce que c’est une forme d’expression éthique et esthétique par laquelle je peux me réaliser, parce que je recherche la liberté totale du corps et de l’esprit.
Et parce que ça me plaît »

Patrick Berhault

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Chapter 3)

Abstract

PROTEIN UNFOLDING ON INTERFACES: A STRUCTURAL AND FUNCTIONAL STUDY

The spontaneous adsorption of protein molecules on interfaces is an ubiquitous phenomenon in natural and man-made systems. This phenomenon plays a central role in many fields, such as health, food, environmental science, and biochemical or immunochemical analysis. The structural rearrangement caused by the direct contact with the sorbent phase may affect protein biological activity, including bioavailability, and ability to bind micro- and macromolecular ligands. Moreover, protein immunoreactivity has been assessed to change if protein molecules interact with a hydrophobic phase; indeed adjutants are hydrophobic substances that act as enhancers in antibodies production.

Whether proteins unfold randomly or through subsequent ordered and eventually reversible steps remains often unknown, and information about the molecular determinants of the "gain of function" or the "loss of function" observed upon adsorption is scarce. The aim of this work is to understand the structural and functional changes that soy storage proteins (β-conglycinin and glycinin) and bovine β-lactoglobulin (BLG) undergo after adsorption on hydrophobic nanostructured surfaces, in our case oil-in–water nanoemulsion and 46 nm polystyrene nanoparticles.

In this three year of my PhD program I deeply investigated the structure of three proteins (BLG, β-conglycinin and glycinin) when adsorbed on different hydrophobic surfaces (oil in water nanoemulsions and polystyrene nanoparticles). I also addressed the changes of biological activities, with a special focus on BLG.

Soy proteins are one of the most attractive plant food proteins. Glycinin and β-conglycinin are the major soybean storage proteins and constitute the 40 and 30%, respectively, of total soybean proteins. Glycinin is a heteromultimer, with a molecular mass of 300-380 kDa. It consists of the intermediary subunit, in which one acidic and one basic subunits are linked via disulphide bond interacting non-covalently to give a hexamer. β-conglycinin is a glycosylated hetero-trimer composed randomly by three different subunits: α (≈67 kDa), α' (≈71 kDa) and β (≈50 kDa). The α and α' subunits are composed by two different domains: the core region and the extension region. The β subunit only contains the core region.

β-lactoglobulin (BLG) accounts for ≈ 65% of the total whey proteins in milk. BLG is a globular protein consisting of a single polypeptide chain composed of 162 amino acid residues and a molecular mass of about 18.3 kDa. It belongs to the lipocalin family, and its three dimensional structure is characterized by a central β-barrel composed by 8 β-strand and a α-helix in the C-terminal tail. This protein contains two disulphide bonds (C_{66}–C_{160})

and (C_{106}–C_{119}) and one free cysteine group (C_{121}). It is present as a monomer at pH < 3, while at neutral pH it exists mainly as a dimer. It can bind and transport hydrophobic molecules in its central hydrophobic pouch, but at now its function is not completely understood.

Protein conformational changes after adsorption on interfaces were evaluated by using different techniques, including fluorescence and solid-state fluorescence spectroscopy, CD spectroscopy, along with limited proteolysis followed by recognition of released peptides by MS. Moreover, changes in biological behavior were evaluated by measuring changes in immunoreacivity that may be relevant from the standpoint of immune response or immunomodulation. Experiment aimed to evaluate the influences of interface denaturated protein on live cells was carried out. For this purpose BLG and BLG-stabilized emulsions, both labeled with FITC, were incubated with monocytes and differences in protein uptake were evaluated by citofluorimetry. In order to have a model of BLG denaturation on the polystyrene interface, an *in silico* study was performed. The simulation was carried out using the computational suite MOE (Molecular Operating System).

In my work structural changes of β-conglycinin and glycinin in solution were compared to those occurring when the proteins are adsorbed at the oil-water interface. Both proteins undergo structural modifications after adsorption on the oil droplet surface. From the standpoint of protein chemistry, the modifications occurring at the interface with the proteins investigated here have some peculiar traits, in what both these proteins expose their tryptophan-containing extension regions to the aqueous phase rather than to the droplet interior, as observed for other proteins. It is very important to note that, in β-conglycinin, tryptophans are present in the extension domains of α and α' subunits, and the present fluorescence data confirm previous results demonstrating that the polar extension regions in these proteins are important for their emulsifying ability. These results support the hypothesis that while the α and α' core domains interact with oil phase; the extension regions protrude into the aqueous phase and stabilize the emulsion droplets by providing the necessary polar regions. Also glycinin's tryptophans containing regions are exposed to the aqueous phase. However, the multiplicity of glycinin's genetic variants makes it much more challenging to derive definite answers from the hydrophobicity profiles of this protein, and some more detailed proteomic work is needed to better understand which portion of the protein anchors to the interface. It is also interesting to note that heat treatment does not affect the structural features of either protein once they are adsorbed at the oil-water interface. In other words, the modifications occurring upon adsorption at the

interface appear to “lock” the protein structure in a conformation that is insensitive to further physical denaturation, at least under the temperature/time regimes employed in this work. As a matter of fact, it is somewhat expected that, in emulsions, the structural regions more sensitive to the entropic changes ensuing from alteration in the water structure (i.e., the protein hydrophobic core) are at least partially buried into the non-polar lipid phase, and thus are insensitive to temperature-dependent changes in the colligative properties of the solvent. The various peculiarities of these systems and their practical relevance seem worth further investigation. We are currently addressing the molecular details of the observed events, in an attempt to identify specific molecular determinants of the different behavior of these proteins, as well as the changes occurring during heating, and to assess whether the conformational changes reported here result in biologically relevant modifications when emulsions are consumed as food.

Also BLG structure changes after interaction with a hydrophobic interface. The intrinsic fluorescence spectrum of adsorbed BLG is red-shifted compared with the free-protein one thus indicating that the adsorbed protein assumes a new structure in which Trp_{19}, usually buried inside the hydrophobic core, is exposed to water. Moreover, adsorbed BLG increases ≈2 folds its global quantum yield. This phenomenon could be explained either by the moving of Trp_{61} away from the Cys_{66}-Cys_{160} disulphide bond, and/or by the moving of Trp_{19} from Arg_{124}, thus removing fluorescence-quenching interactions within the protein structure. The only free thiol in BLG is on Cys_{121}, which is buried in the native structure, but becomes readily and almost completely accessible after adsorption. The overall BLG surface hydrophobicity seems to increase after interaction with the hydrophobic surface, confirming the occurrence of major rearrangements. BLG sensitivity towards trypsin – and therefore the resulting peptidic pattern - is modified as a function of the hydrophobic support where the protein is adsorbed. In fact, in the case of NP-adsorbed BLG trypsin resistance is similar to the one of free BLG, whereas it dramatically decreases for emulsion-BLG. All these data demonstrate an extended stretch of the native structure after adsorption on hydrophobic surfaces with the exposure of new protein regions usually buried from the aqueous media.

Changes in immunoreactivity occurred after adsorption on hydrophobic surfaces. BLG adsorbed on oil droplet surface is more reactive (≈35%) than the free protein by using the 5G6 MAB, and also it is more reactive (≈110%) when using the 1E3 MAB. BLG adsorbed on latex NP is likely to increase its immunoreactivity by using both MAB, that indicates that BLG assumes different structures as a function of the interacting interface(s). We could also hypothesize that the adsorption on the hydrophobic surface

does not increment the number or the type of exposed epitopes, but it locks the protein structure able to bind more efficiently the antibodies.

Cells experiments show how the BLG internalization by monocytes follows two different kinetics according to protein physical state. Moreover the absorption of adsorbed BLG seems to be not influenced by competition of free BLG, leading us to hypothesize the presence of two different pathways for the protein internalization depending on their physicals state.

The *in silico* denaturation simulations demonstrate that the interaction orientation is fundamental for the type and magnitude of protein structure reorganization. Only one pose shows a broad structural reorganization. Other poses show small structural modification, but some starting points of unfolding seem to appear. Longer simulations will give us a more complete overview on this phenomenon. The system, all build by us, seems to be very stable, and the latex denaturating interface should be used with others proteins.

In conclusion in this thesis I described in deeply the structural modification that three proteins, whit a huge importance for nutrition and food science, undergo after adsorption on different model hydrophobic interfaces. I also produced an *in silico* model for computational prediction of protein denaturation on polystyrene interface. Physiological implications regarding protein structural reorganization were also explored.

Chapter 1)

A GENERAL INTRODUCTION TO PROTEIN ADSORPTION ON HYDROPHOBIC SURFACE

Overview on protein structure

Since Anfinsen's experiments in the 1960s, it has been believed and today generally accepted that folding and the resulting native structure of proteins are autonomously governed and determined by the amino acid sequence of a particular protein and its natural solvent environment. The function of a protein can only be interpreted from its structure. A linear polypeptide chain is autonomously organized into a space filling, compact, and well defined three dimensional structure. In a globular protein, the internal core is mostly formed by hydrophobic amino acid residues, held together by van der Waals forces, and the surface of the globule is formed by mostly charged and polar side chains. Proteins exist in this state of condensed matter while the specific conformation is largely determined by the flexibility of the polypeptide backbone and by specific, intermolecular interactions among the amino acid side chains.

The native conformation could be energetically stable or thermodynamically stable. From a thermodynamic point of view, the free energy of a protein molecule is influenced by the following major energetic contributions: (1) hydrophobic effects, (2) hydrogen bonds, (3) electrostatic interactions, and (4) the conformational entropy due to the restricted motion of the main chain and the side chains. The hydrophobic effect used to be explained as a primarily entropic effect arising from the rearrangement of hydrogen bonds between solvent molecules around an apolar solute. This hydration process is energetically unfavorable, and therefore drives apolar solutes together, thereby decreasing their solvent exposed surface area. Today, the hydrophobic effect is usually viewed as a combined effect of hydration (an entropic effect) and of van der Waals interactions between solute molecules (an enthalpic effect) (Makhatadze and Privalov, 1995). It is therefore entropic at low temperatures and enthalpic at high temperatures, which results in a complex temperature dependence of its strength (Schellman, 1997).

Nevertheless, the hydrophobic force has long been considered as the major driving force of protein folding (Dill, 1990) as it leads to a rapid collapse of the polypeptide chain, thereby largely reducing the configurational space to explore. With no doubt, hydrophobic interactions are also a major stabilizing force contributing to the thermodynamic stability of the folded state. The role of hydrogen bonds in folding and stability used to be underestimated based on the argument that intramolecular hydrogen bonds can be replaced by hydrogen bonds between the protein and the solvent. After a number of mutational studies, however, hydrogen bonds have now been recognized as having a contribution to some extent protein stability as important as the hydrophobic effect (Pace *et al*., 1996).

This contribution was estimated to be 1.5-1.0 kcal/mol per buried intramolecular hydrogen bond (Pace *et al.*, 1996). Electrostatic interactions such as ion pairs and salt bridges in proteins have been an area of active research (Kumar and Nussinov, 2002). Whereas hydrogen bonds and hydrophobic forces are essentially nonspecific, electrostatic interactions are largely specific and therefore play an important role in establishing the protein fold of a protein, as well as in determining protein flexibility and function.

Computational and experimental evidence shows that salt bridges can be stabilizing or destabilizing. On the other hand, genome - wide and structural comparisons of thermophilic and mesophilic proteins indicate that salt bridges may significantly contribute to the enhanced thermal stability of proteins from thermophilic organisms (Szilagyi and Zavodszky, 2000; Li *et al*., 2005; Razvi and Scholtz, 2006). The major destabilizing contribution to the stability of the folded state is the conformational entropy of the polypeptide chain. Folding a long chain into a specific, compact structure obviously results in a significant entropy decrease. This is counterbalanced by the various intra-chain interactions described above. The resulting overall stability of the protein (the free - energy difference between the folded and the unfolded state) is marginal, being on the order of 5–10 kcal/mol. This number is a small difference between huge stabilizing and destabilizing contributions. We qualitatively know that the hydrophobic effect and hydrogen bonds are the major stabilizing contributions and the conformational entropy is the major destabilizing one.

All these assumptions are true for physiological, aqueous media, but if agents able to disturb and modify the forces that stabilize the overall protein structure are present, proteins can undergoes structural changes. Among this agent, we found ionic strength, pH, chaotropes and agents able to perturb the entropic boost, as are hydrophobic interfaces.

Interfaces

By definition, an “***interface”*** (i.) is the thin region that separates two phases (two liquids, a solid and a liquid or two solid). The term ***"surface"*** refers only to the region that separates two phases, one of which is gaseous, but the two words are regularly used interchangeably, given the many similarities (Myers, 1999).

The phases can be formed by different types of molecules or by different physical states of the same molecule and substances with a marked affinity for the i. may be present as solutes or insoluble adsorbed layer deposited on them. The type and concentration of molecules at the i. dictate the structure, size, free energy, electrical and rheological properties of the i. and are therefore fundamental in the characterization of a

multiphase system.

There are several types of i., each been of interest for specific technologies. For example, liquid/gas i. (Figure 1.1) are interesting in studying the stability of foams, those between solid and liquid are investigated to understand the mode of action of detergents, adhesives and lubricants. Solid/gas i. are responsible for processes regarding adsorption, catalysis and contamination.

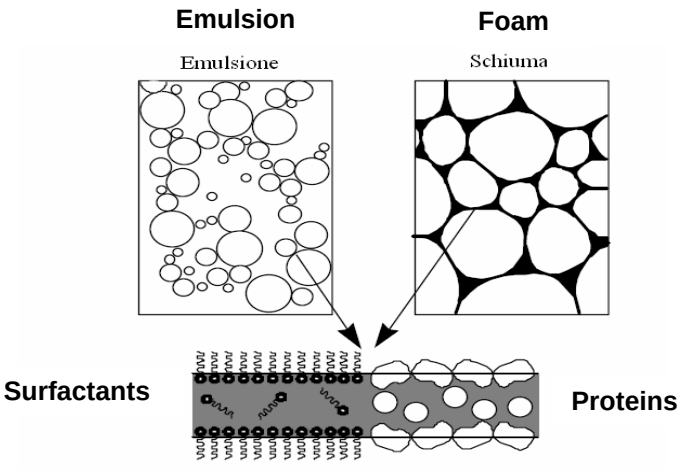

Fig 1.1: *surfactant and protein disposition on emulsion and/or foam interface*

Each molecule in a multiphase system has its own function. In foams, in order to increase the stability of the dispersion of the bubbles and prevent coalescence, it is good to increase the elasticity and thickness of the surface adsorbed state by promoting the formation of protein films. In emulsions, however, it is fundamental to minimize the electrostatic repulsion, in order to stabilize the adsorbed layer and prevent flocculation (Golding, 2004). The surface tension, defined as the property of a surface to resist to an external force, is so much higher in thin adsorbed layer and the thickness can be increased with the adsorption of additional surfactant or by migration of surfactant molecules from areas with a lower surface tension to the areas of reduced thickness.

Since the physico-chemical properties of the medium are also dictated by the nature of the i. it is important to understand the factors that most affect their composition. Type and amount of absorbed molecules are the first, but one should also check the type of the medium, temperature, pH, ionic strength, and all operating conditions.

For example, for globular proteins, increasing their concentration in the medium, increases the amount of adsorbed molecules, because the major interactions lead to the formation of aggregates faster than the single absorbable protein. Temperature also plays an important role: denaturated proteins promote the formation of aggregates. Conditions of

pH near the isoelectric point and high ionic strength have the same effect of temperature by reducing or removing electrostatic repulsion between similar proteins.

Very frequently, the i. are composed by different types of molecules and for this reason it is necessary to consider the single speed and mode of adsorption, the chemical and physical properties and possible interactions. In the case of mixed systems, the i. is initially formed by molecules that adsorb more rapidly. With the arrival of the other the i. evolves until it reaches an equilibrium in which the various system components, including those present in the medium coexist in dynamic equilibrium.

Hydrophobic effect

To better understand the mechanism that drive adsorption of protein on interfaces and their structural reorganization it good to introduce the physical phenomenon called “hydrophobic effect”, defined as the unaffinity of non polar compounds such as hydrocarbons towards polar solvents, like water. In molecular terms the process leading to this unaffinity is complex and can be described on chemical-physical basis.

The non-polar molecules are unable to compete with the strong attraction between the molecules of polar solvent, due mainly to the presence of hydrogen bonds between water dipoles that are stronger than the intermolecular interactions that attract apolar parts each other. The hydrogen bonds are characterized by high directionality; they are, in fact, stronger when the molecules involved in the bond are oriented in such a way as to maximize the electrostatic interactions and this occurs when the hydrogen atom and two electronegative atoms are aligned.

In a polar solute, such as water, the dissolution of charged or polar molecules is facilitated by the formation of hydrogen bonds between solvent and solute and the ability of a dipolar solvent, such as water, to orient its dipole due to the charge of the solute. In thermodynamic terms the presence of a charged or polar molecules in water results in an increase in entropy of the system with a favorable change of free energy, where there is a slight increase in enthalpy related to the modification of hydrogen bonds offset by a greater increase in entropy.

Hydrophobic solutes do not cause interactions with the polar solvent and their addiction leads to an increase in enthalpy, requires the breaking of hydrogen bonds energy intake from the environment. In addition, the dissolution of molecules hydrophobic leads to a decrease in entropy. A model that can explain the behavior of solutes in a nonpolar polar solvent like water has been identified in the "iceberg model". It proposes that a non-polar solute lead to the creation of a "cage" of a layer of

ordered water around it. In this structure the water molecules can not build bridges hydrogen to the solute, thus forming a "fence" welded by hydrogen bonds around the solute (Figure 1.2).

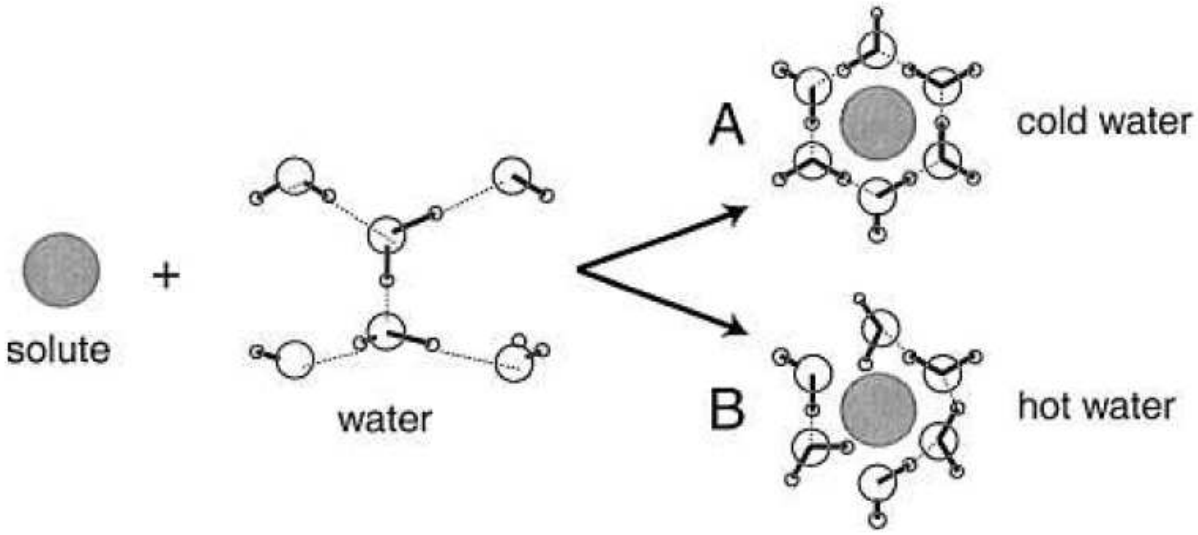

Fig 1.2: *"Iceberg" model for apolar solutes in water. At low temperatures (A) molecules water surrounding a solute in non-polar, assume an ordered structure (low entropy) for maximize the entropy of the system. At high temperatures (B) the water molecules increase the state of disorder.*

To minimize the loss of entropy of the system, a number of polar solvent molecules are then ordered (as few as possible of course). The number of ordered water molecules and therefore the extent of the decrease in entropy, is proportional to the hydrophobic surface exposed by the solute. The free energy change due to the presence of a solute in a polar solvent is therefore unfavorable for a positive value of ΔH and a decrease of ΔS. The hydrophobic effect is characterized by negative entropy at low temperatures and by a negative enthalpy at high temperatures. In general, low-temperature thermodynamic processes are characterized to decrease their enthalpies, whereas high temperatures have led to states of high entropy. In the iceberg model the increase in temperature causes an increase in orientation distribution of water molecules that constitute the first layer around the solute in order to gain entropy. This leads to breaking of bonds hydrogen molecules between the first layer of the same with the result of an increase in enthalpy.

The measure of hydrophobicity of a given chemical species can, at least from the conceptually point, can be expressed as an equilibrium distribution. A solute shows a certain chemical affinity for each phase: polar and nonpolar.

This affinity is defined as μ°, where μ° = h° - TS°. The solute also has an entropy of transition at each stage, represented by KTlnC, where C is the measure of the concentration of the solute in that phase.

The distribution of the solute between the two phases reaches equilibrium when the difference equals the difference of chemical affinity and concentration is obtained as a result that:

$$\Delta\mu^{\circ} = -KT \ln C2/C1$$

And, simultaneously:

$$\Delta\mu^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ}$$

For simple systems, such as non-polar solutes in nonpolar solvents, the change in entropy is small and ΔH° is the main component of Δμ°. The free energy of transfer of apolar molecules in a polar solvent is positive and large ($\Delta\mu^{\circ} >> 0$).

In terms of free energies, Δμ° is maximum at a temperature close to the point of boiling water, indicating that the interaction is less favorable at that temperature. However the experimental tests have shown that the solubility of a solute polar in water is proportional to Δμ°/KT and that the hydrophobic effect is greatest in at room temperature. The term "hydrophobic effect" refers to the insertion of a single non-polar solute in water, with the words "hydrophobic interaction" refers to the association of two parts of polar molecules in water. The distinction stated motivates the definition of two characteristic temperatures: T_s, where Δμ° is maximum and ΔS°≈0, and T_h, where the enthalpy of transition is minimal and the ratio Δμ°/KT is maximum.

The simplest view, which assumes that the cost in free energy for creating a "cavity" in which to insert the solute depends exclusively on the surface of the cavity itself, two apolar solutes are driven to associate in water to reduce surface area of the solute-water contact. The interaction of two non-polar solutes in water is described by the *"potential of mean force "* (p.m.f) (Figure 1.3).

As function of the distance there are two minims that indicate the two energy-favorite states: the first where the two solutes are in contact, the second where the two solute molecules are separated by a water molecule. Intermediate stages between these

two are disadvantaged. Through a discussion of hydrophobic effect in thermodynamic terms, although short, strong ability to perceive the structuring of water in biological systems through a force of entropic nature that leads to non-covalent interactions of molecules, or parts of them, they are able to interact directly with water.

The hydrophobic effect, such as hydrogen bonds and van der Waals interactions are weak if considered individually, but being present in large numbers in the systems they play a major role in the three-dimensional structure of proteins, nucleic acids, polysaccharides and lipid membranes, also due to the nature individual cooperative interactions within complex polymeric structures.

Adsorption of proteins on hydrophobic interfaces

The incompatibility of no polar compounds such as hydrocarbons, with respect to polar solvents such as water, was defined above as hydrophobic effect.
The non-polar molecules are unable to compete with the strong attraction between the molecules of polar solvent, due mainly to the presence of hydrogen bonds between water dipoles that are stronger than the intermolecular interactions between the apolar parts that attract them. Hydrophobic solutes do not cause interactions with the polar solvent and their introduction leads to an increase in enthalpy, the rupture of hydrogen bonds requires the intake of energy from the environment. In addition, the dissolution of hydrophobic molecules causes a decrease in entropy (for example, associated with the organization of water molecules) to minimize this loss of entropy. The systems tend to "hide" apolar solutes, organizing the fewest number of molecules 'water around them, or in the case of complex molecules with an inherent flexibility, to fold into structures that are not accessible to solvent. As mentioned above, this phenomenon is called "hydrophobic effect" and plays a decisive role - along with other “weak” chemicals bonds - in the definition of the three-dimensional structure of proteins, nucleic acids, polysaccharides and lipid membranes, also thanks to the cooperative nature of individual interactions within complex polymeric structures. The phenomenon of protein adsorption to an interface plays a major role in many natural and artificial systems. Is well known that proteins adsorbed to an interface such as oil/water emulsions, and they can be stabilized by these biomolecules. The conformational changes that proteins undergo during the process of adsorption mainly affect their function and properties. However, knowledge about these structural changes, such as the possible formation of polymeric states, is still very limited. But the complexity of systems in which non-aqueous and aqueous phases coexist together makes it difficult to study structural changes of the protein in question.

There are many other cases of systems, in addition to that concerning food systems where protein adsorption is a phenomenon of considerable importance. An example of the adsorption process essential to the life is the statherin (STATH) case. Statherin is thyrosine rich phosphopeptide that plays a key role in the growth of bone tissue in the human body. This protein is adsorbed to the surface of bone hydroxyapatite (Gilbert *et al*., 2000), and its binds to the surface and recognition are essential for the functioning of this protein.

Chromatography and analytical techniques are examples of artificial methods that exploit the properties of affinity between the proteins and surfaces of certain matrices (Chase, 1994; Regnier, 1987). The chromatographic techniques are very successful in the field of protein purification and require a great knowledge of the optimal conditions of pH, ionic strength and temperature to control the proper absorption. In fact, during this phase, the structure of the protein in question (from primary to quaternary), plays an important role in determining whether or not the protein will binds to a given matrix (Regnier, 1987).

The field of nanotechnology, which in recent years has seen increasing interest and has been the subject of an exponential growing numbers of studies, provides other important examples in which the process of protein adsorption plays a significant role. The proteins can be adsorbed on smooth surfaces such as through the technique of lithography (Lee *et al*., 2002). These properties can be exploited in proteomic studies or in the screening process of pharmaceutical products.

However, the process of adsorption of a protein interface can also be an undesired phenomenon. There are several cases in which this event is best avoided. An example are the proteins adsorbed on the materials used for the production of artificial prostheses implanted in the human body. These materials, such as bone implants, are exposed to body fluids that contain protein molecules that immediately adhere to their surface (Andrade *et al*., 1986). It possible that the structural changes of adsorbed proteins can indirectly cause adverse reactions after implantation of foreign material to the body, such as inflammation and thrombosis (Balasubramanian *et al*., 1999, Hu *et al*., 2001). Another example of unwanted protein adsorption to human health is the well-known case of fouling of contact lenses (Furness *et al*., 1998). The daily cleaning of contact lenses with a detergent is required so that it prevents the accumulation of adsorbed proteins causing loss of product performance.

Driving forces of protein adsorption on interfaces

The '"hydrophobic effect" is generally recognized as the driving force that guides the process of folding in a protein (Dill, 1990). In this scenery, the residues that show a more

hydrophobic part in the polypeptide chain are hide in the native protein from the aqueous environment. The hydrophobic interactions thus assume a role of considerable importance in the formation of tertiary and quaternary structures of proteins.

The interaction between apolar structures is in fact considered to be of high importance in the associations between proteins, although we can not ignore the contribution arising from electrostatic energy. Protein/protein interaction sites at the interface show a high density of charged residues compared with the rest of the surface (Tsai *et al*., 1997). The evaluation of the composition in amino acids present at the surface (Tsai *et al*., 1997) shows that, while the hydrophilic amino acids, and polar loads, are more present in the surfaces compared to the hydrophobic core of the structure, they are not as frequent as expected over the entire surface. The effect of hydrophilic residues engaged in stabilizing molecular interactions is more significant than that which they exercise in the protein core and is therefore important to assess the relative contribution of these effects (Jones & Thornton, 1996).

The difference between protein folding and protein interaction in terms of hydrophobicity can be estimated by basis according these aspects (Tsai *et al*., 1997). The primary motivation of a different degree of hydrophobicity can be found in the folding process itself: first the polypeptide structure has a greater degree of freedom and can adopt a large number of potential configurations, the folding polypeptide chain can be found to maximize the hydrophobic interactions, whereas the association between proteins already folded into a stable form involves a structure with a little freedom to alter its conformation in order to reach a greater extent of interactions between nonpolar residues. The absence of strictly hydrophobic areas on the surface of the protein and the presence of different number of polar residues can be seen as a compromise between achieving high stability of an interface and its possible existence in thermodynamics terms.

The forces that determine the adsorption phenomenon of proteins to solid interface (Lee *et al*. 2001) are of different types and include van der Waals forces, electrostatic interactions, solvation energy and entropy effects (Oscarsson, 1997). The extent of adsorption is determined by the competition between attractive and repulsive forces, as shown in Figure 1.3

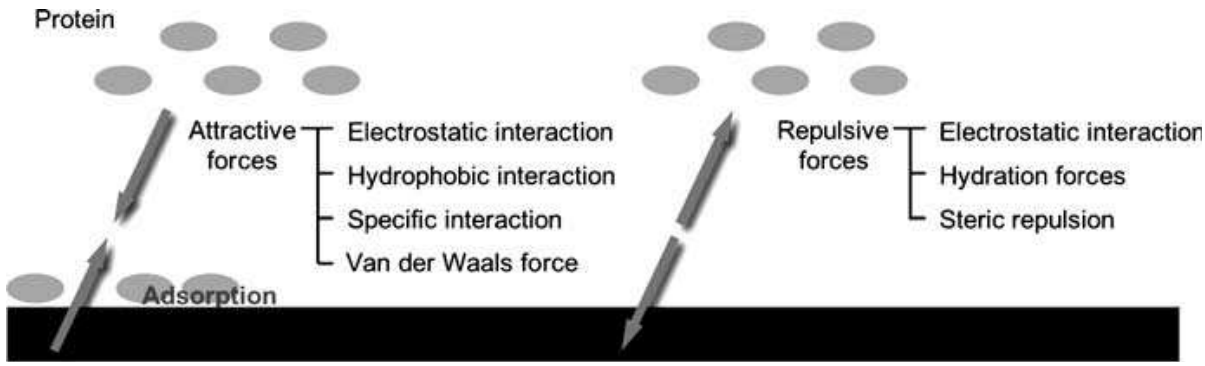

Fig 1.3: *attractive and repulsive forces involved in protein interface adsorption. From Y. Iwasaki et al. 2001*

Among the existing forces, van der Waals forces and electrostatic ones play an important role, while others are often consequences of structural or conformational changes of the protein. However, electrostatic interactions do not seem to play a role with regard to the surfaces of biomaterials that are in the most of the case essentially free of charge. In addition, the surface of these matrices generally do not have structures that can be recognized by molecules with a specific tertiary structure, as the binding sites of antibodies and enzymes. Therefore in the absence of such forces hydrophobic interactions hold the major role in the stabilization of these novel structures.

Protein behavior after adsorption

The process of nonspecific adsorption of globular proteins to a solid interface induces changes in protein structure in relation to the nature and size of the adsorbent material and tot the structural stability of the protein in question. It is well known that proteins can adopt different three-dimensional structures under the influence of "perturbing agents". In this context, the external contact with a surface could stabilize or prejudicing the presence of certain conformers, which then influence the kinetics of absorption and desorption. In recent years a number of studies have been made with the aim to investigate what are the forces that govern the process of adsorption at a solid interface (Norde 1998, 2000).

A common feature of globular proteins is to expose polar residues below the surface of the molecule, while most of the apolar amino acids are "buried" in the hydrophobic core. However, a fraction of the protein surface is characterized by the presence of hydrophobic regions (Branden and Tooze 1991; Richards 1997).

The spontaneous adsorption of protein molecules to a solid surface can occur if the Gibbs energy of the system decreases. The contribution to the increase in enthalpy of the

system is given by electrostatic interactions and hydrogen bonds, while the change in entropy is derived from the change in the order of the initial structure of the adsorbed protein, and by the rearrangement of water molecules at the interface.

The process of the protein unfolding results from a number of elements in the system. The denaturation of globular proteins occurs when the system Gibbs energy is equal to 20-100 kJ mol^{-1}, which is the equivalent energy required for the destruction of 1-8 hydrogen bonds. However, these interactions do not involve the entire protein and native areas partly coexist in the structure (Radford *et al*. 1992). During a denaturation process in solution the energy change is determined by the increase in enthalpy and entropy loss due to the reorganization of water molecules around the protein amino acid with the new arrangement exposed to the solvent. The protein exposes portions to the solid matrix that are usually hide in the native state. Consequently, the mechanism of adsorption of a protein to solid interface is different from those caused by protein denaturation induced by the addition of substances in solution or by heat denaturation.

There are several cases to consider in the process of adsorption of a protein interface. First of there is the case in which the outer surface of the protein is polar as the matrix which adsorbs. Here are a few water molecules are retained to solvate charged residues of the protein to the surface. If the protein surface is quite polar and the adsorbent phase is polar, or vice versa, there is a dehydration of the protein-solid phase interface. If both protein and solid surface have distinct characteristics of hydrophobicity, we assist to a protein structural reorganization and to a global entropy modification (Boulkanz *et al*. 1997; Dorsey and Dill 1989, Gilpin 1993, Lu *et al*. 1998; Norde 1998). In some cases the adsorption to a hydrophobic surface can induce conformational changes important enough to cause an increase in hydration of the protein (Boulkanz *et al*. 1995; McNay and Fernandez 1999).

In any case the process of adsorption to an interface of a protein depends largely on the chemical and electrical properties of the adsorbent matrix. Several studies on the proteins adsorption to a polar and charged support show that most of the forces that govern the process are electrostatic (Gill *et al*. 1994; Lesins and Ruckenstein 1989; Quiquampoix *et al*. 1995; Servagent-Noinville *et al*. 2000; On *et al*. 1998b). For this reason many studies have focused on the development of models with interfaces electrostatically neutral. In this case, the different properties of hydration of both protein and sorbent matrix determine the repulsive forces that inhibit the process of adsorption (Herrwerth *et al*. 2003; Jeon *et al*. 1991). Using hydrophobic supports, the adsorption process is determined by interfacial energy reduction and by the replacement of water molecules at the interface

with proteins (Vogler 1998).

In addition it is useful to draw attention to the interactions between proteins in the system, both or either electrostatic or hydrophobic in nature, which could cause aggregation of proteins at the solid-liquid. In both cases these forces govern the orientation and the specific structure of the adsorbed protein molecule (Malmsten, 1998; Wahlgren *et al*. 1998), as shown in Figure 1.4.

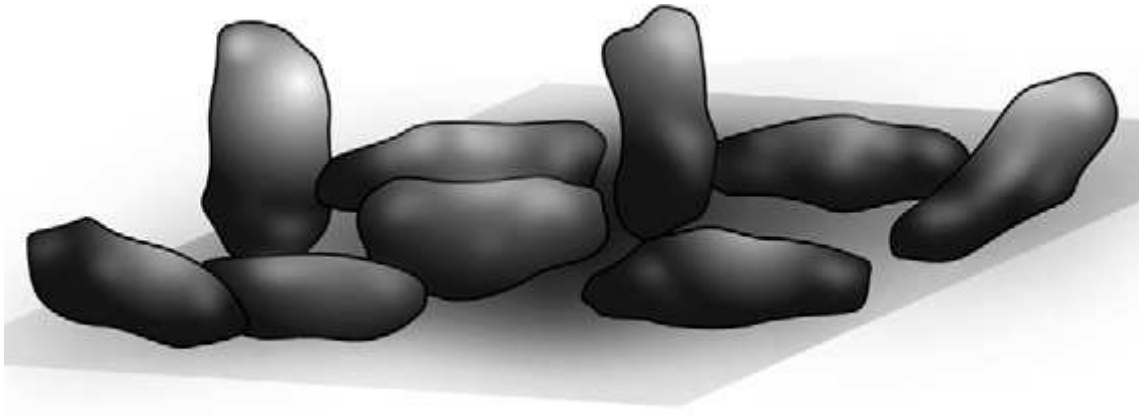

Fig 1.4: e*xample of monomolecular layer of globular proteins adsorbed on a solid interface. From Norde W., 2008.*

Almost all the adsorption process are characterized by the lack of specific knowledge in terms of energy and entropic contributions to the system in terms of:

- Redistribution of charged groups (ions) when proteins are superimposed on the surface
- Dispersion of forces between the protein and the adsorbent
- Change in hydration of the solid surface of the protein
- Structural rearrangements of the protein molecules

In general, the process of adsorption of proteins from an aqueous solution to a solid support involves three main steps, as shown in Figure 1.5.

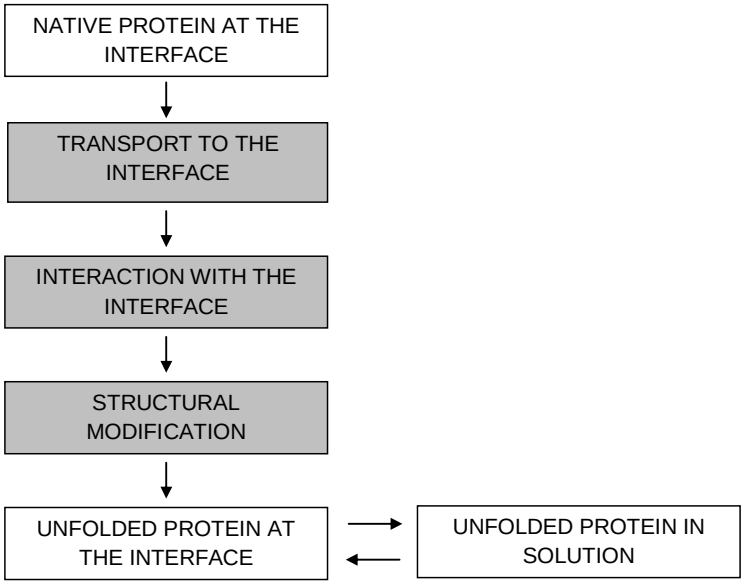

Fig 1.5: s*chematic of a model denaturation process at the interface*

In many circumstances the forces that govern the process of adsorption have their origin from hydration or dehydration of a hydrophobic surface phenomena that are much stronger than electrostatic contributions (which vary according to conditions of pH and ionic strength of the system). For this reason it is expected that all proteins can adhere to apolar surfaces even in unfavorable electrostatic conditions.

The behavior of a protein at the interface also depends on its structure. There is a classification on the globular proteins, which distinguishes them according to their stability during adsorption (Arai and Norde 1990, Kondo *et al*. 1991; Norde 1991). These authors introduced the concept of "soft" and "hard" protein. Proteins classified as "hard" undergo small structural changes after adsorption and adhere to surfaces only if attracted by electrostatic forces. "Soft" proteins instead undergo more changes in the structure, resulting in a conformational entropy change necessary for the protein to engage the polar surface (Figure 1.6).

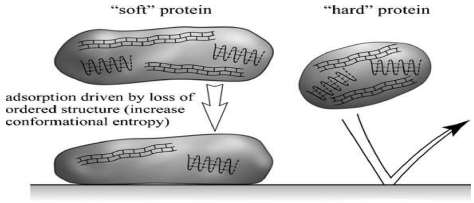

Fig 1.6: *protein classification from their attitude to interact with a non polar surface. Form*

Norde W., 1998

There are several causes of the change in the protein structure when bounds to a solid interface. One of these is the possibility or impossibility of forming hydrogen bonds with the polymer matrix. In aqueous environment the globular structure of a protein is strongly determined by hydrophobic interactions, this means that the protein hides his polar portion from the water. The hydrogen bonds between polypeptide units in the protein stabilize the formation of ordered structures such as α-helices and β-sheet. But when the protein adsorbs to the solid surface, a part of it was first exposed to the solvent and then is put in contact with the solid interface. As a result the intramolecular hydrophobic interactions decrease in intensity and they are no longer the main factor involved in the stabilization of the molecule. The apolar portions, which in aqueous solution are hidden inside the protein, could be partially exposed to the surface without coming into contact with water. This behavior depends from the hydrophobicity of the solid surface.

Polar surfaces could interact via hydrogen bonds. Therefore, peptide sequences, which lose their spatial conformation when portions of α-helices and β-sheets are destroyed, they can form hydrogen bonds with the surface. However, despite these portions are anchored to the surface, a substantial loss of the original ordered structure could increase the entropy of thesystem.

Apolar surfaces have no opportunity to interact via hydrogen bonds with the protein. The process of adsorption of a nonpolar matrix could stimulate the creation of hydrogen bonds between the polypeptide chains in the interfacial region of the protein so as to promote ordered structures in the protein molecule.

Protein molecules spontaneously adsorb to a surface when the corresponding energy of adsorption, ΔG_{ads}, is negative. It is been shown that the major contribution to a negative ΔG_{ads} derives from the presence of hydrophobic and electrostatic interactions. However, the conformational changes also contribute to decrease ΔG_{ads} thus providing a further boost protein adsorption (Norde and Lyklema, 1991; Norde, *et al*. 1995; Norde, 1999). The first observations showed that structural changes of the protein to an interface, date back almost 40 years ago (MacRitchie, 1972) and were based on measurements of enzymatic activity of proteins adsorbed at the air/liquid. Knowledge of these structural changes is important and essential to understand and to control the adsorption phenomena, as well as to be able to develop the theory real for that phenomenon.

One considerable importance aspect during the adsorption process is related to the “adaptability” of the protein when placed on a surface. The first evidences of this behavior have been disclosed when sophisticated tools and methodologies have allowed to

investigate directly the structure of adsorbed proteins. When a protein adsorbs to a surface loses its structure, yet retains some ordered regions with areas of α-helices and β-sheet. (Norde, 1998).

The protein population present at the hydrophobic interface is characterized by a certain heterogeneity in relation to the state in which the protein is located. A certain amount is in fact present at the surface as a structured protein that retains its native state, while a fraction is present as an intermediate of a simple or complex denaturation passage. Proteins adsorb to the surface and tend to relax its structure in relation to protein concentration: the higher the concentration of protein the less a relaxation of the structure (Figure 1.7).

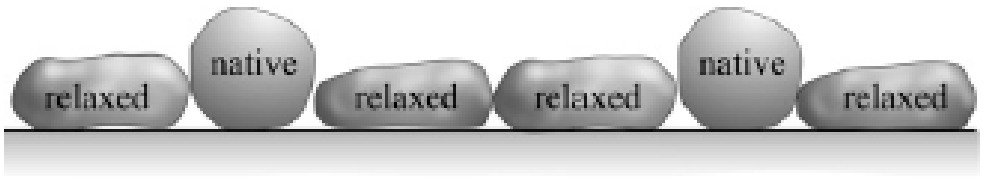

Fig 1.7: *schematics of protein behavior on a general hydrophobic surface. From Norde W., 1998*

The adsorption of proteins on solid surfaces is often described as 'irreversible' since, after attachment is established, which has usually reached a final value within an hour, subsequent replacement of the protein solution by pure solvent, as a rule, does not lead to any significant desorption on a time scale of hours or even days. However, it does not mean that protein molecules remain attached to the surface whatever the conditions of the solution in which the surface is immersed. With respect to reversibility of adsorption/desorption of proteins, distinction should be made between reversibility toward dilution of the solution, changes in pH and ionic strength, addition of other types of surface-active substances and exchange against dissolved proteins. Although desorption upon dilution typically does not occur, protein molecules may be released from the surface by other surface active molecules added to the system through an exchange mechanism in which the protein molecules are replaced from the surface in favor of adsorption of other molecules.

Perhaps, the most clear example of such an exchange process is the "Vroman effect": the transient adsorption of proteins from blood plasma, in which, the more

abundant smaller proteins are displaced by the less abundant larger proteins that have higher affinities for the surface. The exchange process between protein molecules in the adsorbed state and in solution can be heteromolecular, like the Vroman effect, or homomolecular, as in systems where a large amount of one kind of protein is present in excessive amounts so that it is accommodated both in the adsorbed state and in solution.

Then, protein adsorption is a dynamic process by which protein molecules are continually exchanged between the adsorbed and the dissolved states. It is well known that relaxation at the sorbent surface involves more or less perturbation of the original, native protein structure. The question arises whether the molecules returning from the surface into the solution regain their original structure (Norde, 2000).

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Chapter 2)

DENATURATION OF SOY PROTEINS IN SOLUTION AND AT THE OIL-WATER INTERFACE

Introduction

β-conglycinin

β-conglycinin (7S) is a glycosilated hetero-trimer randomly formed by three different subunits: α (≈67 kDa), α' (≈71 kDa) and β (≈50 kDa). The α and α'subunits are composed by two different domains: the core region and the extension region. The β subunit only contains the core region. The core region is well conserved in each subunit and is imputable for the thermal stability of the protein. The extension regions show a sequence identity of 57.3 % and they are strongly acidic (Maruyama *et al*, 2003). Each subunit has specific physico-chemical properties. At pH 7.6 and 0.5 M NaCl the midpoint denaturation temperatures of the recombinant β, α'and α are 90.8° C, 81.7° C and 78.6° C (Maruyama *et al*, 1998). The surface hydrophobicity order of either recombinant subunits is α'>α>β but their emulsifying capacity is in the order α>α'>β (Maruyama *et al*, 2002), and this latter difference has been attributed to the presence of the extended region in the α and α' subunits. β-conglycinin can also form supramolecular aggregates as a function of pH and ionic strength (Than *et al*, 1978).

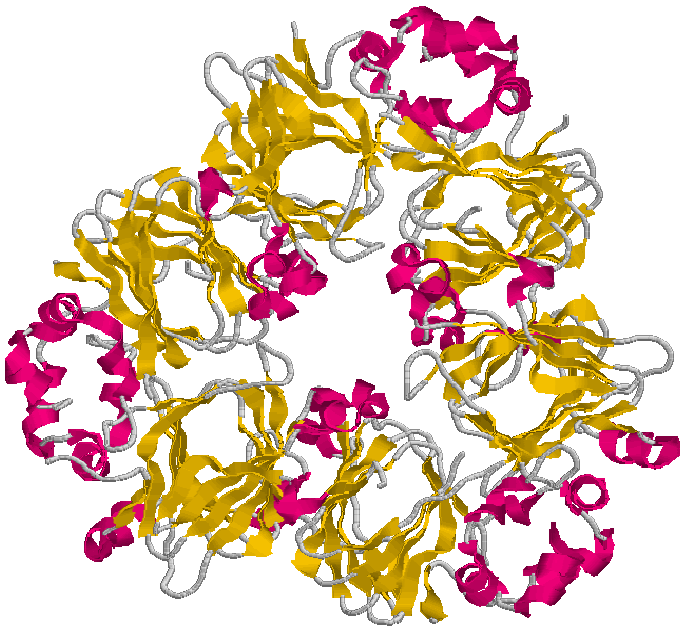

Fig 2.1: *β-conglyicinin α subunit 3D structure (from http://www.uniprot.org/uniprot/P13916)*

Glycinin

Glycinin is a hetero-multimer, with a molecular mass of 300-380 kDa. It consists of two disulphide-bonded subunits – one acid and one basic - interacting non-covalently to give a hexamer that can be dissociated by treating the proteins with chaotropic agents such as urea or guanidinium hydrochloride, or with sodium dodecylsulphate (Shewry, 1995). There are different genetic variants of glycinin (named G1 to G5) that differ also for their thermal stability (Lakemond, De Jong, Gruppen & Voragen 2002). At pH 7.6 and I 0.5 M glycinin has denaturation temperatures ranging from 85° to 9 4° C. (Lakemond, De Jongh, Hessing, Gruppen & Voragen, 2000).

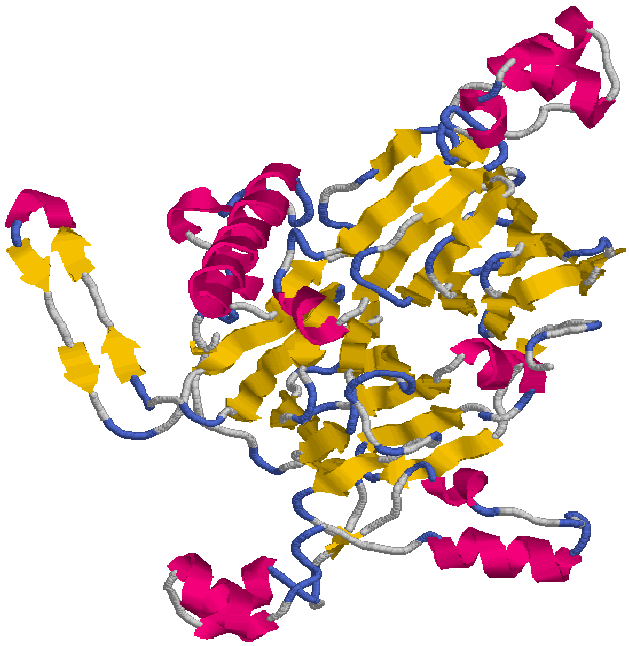

Fig 2.2: *glycinin G2 subunit 3D structure (from http://www.uniprot.org/uniprot/P04405)*

Fluorescence spectroscopy

Fluorescence is a physical phenomenon in which some electrons of specific molecules can acquire a higher energy level from adsorption of photons of appropriate energy, resulting in emission of the absorbed energy in the form of photon energy still high when the electron returns to baseline.

From the second law of thermodynamics, the energy emitted is always less than that absorbed, and therefore the wavelength of emission is greater than the wavelength of excitation. The amount of energy emitted by a specific fluorescent group is also correlated with its chemical and physical-chemical around. Structural proteins organization

informations can be provided by fluorescence measurements on tryptophan residues (Trp), whose indole ring is excitable at 295-300 nm. The fluorescence response may vary depending on the Trp physical surrounding. An observed increase of the wavelength of emission (and therefore a lower energy yield) means that the Trp moves from a non polar environment (for example, the inside of a protein) to a more polar one (for example, the aqueous solvent). The intensity of fluorescence can vary, depending on whether the excited electrons may or may not return to ground state or their energy be lost on other potential acceptors, which are called "quencher".

Spectroscopic techniques for the study of protein structure

Fluorescence spectroscopy is a very powerful tool for studying protein structural changes in complex matrices, such as food products. The fluorescence spectrum is determined by the chemical environment of a fluorescent component (in proteins, it is usually the fluorescent amino acid tryptophan), and therefore, changes in the emission spectra of tryptophan often occur in response to conformational transitions, subunit association, substrate binding, or denaturation of the proteins present in the sample. There is a shift of the maximum of emission to higher wavelength when tryptophan moves from a hydrophobic surrounding to a hydrophilic one (e.g. from the protein interior to the aqueous media). Also fluorescence intensity can change: some molecules can work as quenchers and adsorb the emitted light (Bonomi *et al*, 2004).

Information about protein structural organization may be gathered also by using fluorescent dyes. One of the most popular probes is 1-anilinonaphthalene-8-sulfonate (ANS), that becomes fluorescent when interacting whit a hydrophobic region, making it possible to study protein structural changes in complex systems after a specific treatment (Iametti *et al*, 1993; Cairoli *et al*, 1994; Alizadeh-Pasdar *et al*, 2004; Rasmussen *et al*, 2007; Caldinelli *et al*, 2008).

Traditionally, fluorescence studies have been performed on clear solutions. In the case of solid or cloudy liquid food products (e.g. emulsions) it is possible to apply front face fluorescence techniques (Eisingerand *et al*, 1979; Castelain *et al*, 1994; Rampon, *et al*, 2003; Bonomi *et al*, 2004; Granger *et al*, 2005). In this technique the excitation light beam excites only the first layer of the samples, and the light is emitted from this same surface.

Circular dichroism spectroscopy

Circular dichroism (CD) is a spectroscopic technique based on the absorption of circularly polarized light by intrinsically or extrinsically chiral chromophores placed in an ordered structure. This technique takes advantage of the elliptical polarization of a beam of light, linearly polarized in a plane, suffers after passing through an optically active molecule.

All amino acids except glycine, are optically active, in what they all contain an asymmetric carbon. The interaction between the plane of polarized light and asymmetric periodicals stretches of the polypeptide chain, determines a circular dichroism spectrum attributable to a given conformation. The light source used is usually produced by a xenon lamp that it has continuous emission in the spectral region ranging from 180 to 800 nm.

Aim of the work

At now it is well known that soy proteins readily adsorb at the interface of an oil water emulsion upon homogenization, but very little is yet understood on the details of the structural changes at the interface. The aim of this work is to study the structural changes of soy proteins in solution, with focus on chaotrope and heat-induced changes, and compare it to those at the oil-water interface.

Material and methods

Purification of storage soy proteins fraction

Soy protein isolate (SPI) was prepared by dispersing (1:10 ratio w/v) defatted soy flakes (donated by the Solae Company) in 0.1 M Tris-HCl buffer at pH 8.0. After mixing at room temperature for 1 h, the soluble fraction was separated by centrifugation at 12,000 g for 30 min at 10 ºC (Beckman Coulter, Model J2-21, Fullerton, CA, USA). The centrifuged dispersion was then adjusted to pH 4.8 with 2 M HCl, and refrigerated at 4° C for 2 h. The protein was then precipitated by centrifugation as described above. The supernatant was discarded and the precipitate was washed with 0.01 M sodium acetate, pH 4.8 to a 1:8 ratio (w/v) and centrifuged again. The slurry was resolubilized in ultrapure water (Barnstead International, E-pure^{TM} D4641, Iowa, USA) and the final pH was adjusted to 7.5 with 2 M NaOH. The fraction was dialyzed overnight at 4 ºC, freeze dried and stored at -20° C for further analysis.

The isolation of fractions rich in glycinin and β-conglycinin was carried out as previously described with minor modifications, by suspending the defatted soy flakes (The Solae company) in ultrapure water (Barnstead International, E-pure^{TM} D4641, Dubuque, Iowa) in 1:15 ratio (w/v) and adjusting the pH to 8.0 with 2 M NaOH. After stirring the suspensions for 2 h at room temperature, the insoluble fractions were separated by centrifugation at 9,000 g for 30 min at 20° C (Beck man Coulter, Model J2-21, Fullerton, CA, USA). Sodium bisulfite (0.98 g/L) was added to the soluble fraction adjusted to pH 6.4 with 1 M HCl. After overnight incubation at 4° C, t he protein suspension was centrifuged at 7,000 g for 20 min at 4º C (Beckman Coulter, Model J2-21, Fullerton, CA, USA). A fraction rich in glycinin was recovered in the precipitate, resolubilized with ultrapure water, and adjusted to pH 7.5 with 2 M NaOH. Sodium chloride (0.25 M final concentration) was added to the remaining supernatant, which was adjusted to pH 5.0 with 1 M HCl and stirred for 1 h in an ice bath. After centrifugation at 9,000 g for 30 min at 4° C (Beckman Coulter, Model J2-21, Fullerton, CA, USA), the supernatant was diluted with cold ultrapure water in 2:1 ratio (v/v) and adjusted to pH 4.8 with 2 M HCl. The β-conglycinin rich fraction was then recovered after centrifugation at 7,000 g for 20 min at 4° C. The precipitate was resolubilized with ultrapure water and adjusted to pH 7.5. Both isolated fractions (β-conglycinin and glycinin rich fractions) were dialyzed at 4° C overnight against ultrapure water and freeze-dried. The freeze dried proteins were stored at -20° C.

Preparation of soy protein solution

Both protein preparations (95 % protein on a dry weight basis) were dissolved in 0.05 M sodium phosphate buffer the day before use and stored at 4° C. Before analysis the samples were equilibrated at room temperature for at least one hour. When reported, heat treated samples were prepared by heating protein solutions at 75°C or 95°C for 15 min in a water bath followed by immediate cooling in an ice bath. Urea-treated protein samples were prepared by dissolving the appropriate amount of protein in denaturating buffer (8 M urea, 0.05 M Tris-HCl, pH 7.4) to a final concentration of 1 mg/ml. After at least one hour of gentle mixing, protein solutions were used for the measurements.

Emulsion preparation

Emulsions were prepared as follows: 3 g of soy oil (Sigma Co., St Louis, MO) were pre-emulsified with 27 ml of 10 mg/ml β-conglycinin or 20 mg/ml glycinin solutions prepared as described before, using a dispersing unit (Powergen 129, Fisher Scientific, Ottawa, ON) for approximately 1 min. The pre-emulsion was immediately homogenized through a Microfluidizer (110S model, Newton, MA) for five passes with an overall pressure of 300 kPa. Heating of emulsions was carried out at 75° C for 15 min for the β-conglicinin-stabilized emulsion and at 95° C for 15 min for the glycinin-stabilized emulsion as described above for solutions.

For spectroscopic analysis of proteins in the emulsion, and to minimize the contribution from the unadsorbed protein, the lipid fraction was separated from the aqueous phase by centrifugation (*12.000 x g*) for 20 min using an Eppendorf microcentrifuge. The cream was then removed, spread on a glass fiber filter (Whatman, Fisher Sci), washed with 0.05 M sodium phosphate buffer, and resuspended in 0.05 M sodium phosphate buffer to a final concentration of 10% (w/w). All samples were kept at 4° C until use.

Fluorescence spectroscopy

Tryptophan fluorescence emulsion spectra (λ_{ex}: 280 nm; λ_{em}: 300-450) (Castelain & Genot, 1994; Bonomi, Mora, Pagani & Iametti, 2004) were recorded in a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Corp., Tokyo, Japan) equipped with both liquid sampler holder and front face cell holder. Excitation and emission slits were set at 5 nm. Excitation was at 280 nm to maximize tryptophan quantum yield. Protein fluorescence upon excitation at 280 nm is due to both tyrosine and tryptophan residues. However, the

emission of tyrosine in an aqueous buffer occurs at 303 nm and is quite insensitive to solvent polarity, whereas the emission maximum of tryptophan in water occurs near 350 nm and is extremely dependent upon the polarity of the environment around the sidechain (Lakowicz, 2006). Cream obtained with either β-conglycinin or glycinin was diluted 1:10 (v/v). All spectra are the average of at least three repeated scans.

CD spectroscopy

CD spectra were recorded on a Jasco J810 spectropolarimeter (Jasco Corp, Tokyo) and analyzed by means of Jasco software. Near-UV CD spectra (350-240 nm) were recorded on a 2 mg/ml protein solution in an 1 cm path quartz cuvette (band width: 2 nm, response: 0.25 s, data pitch: 0.1 nm, scanning speed 20 nm/min). Far-UV CD spectra (250e190 nm) were recorded on a 0.2 mg/ml protein solution in a 0.1 cm path quartz cuvette (band width: 2 nm, response: 0.5 s, data pitch: 0.1 nm, scanning speed 20 nm/min). Protein secondary structure estimation was carried out by using CDNN software (http://bioinformatik.biochemtech.uni-halle/cdnn).

Protein surface hydrophobicity and -SH accessibility

Protein surface hydrophobicity was determined by titration with 1-anilinonaphthalene-8-sulfonate (ANS) (Rasmussen, Barbiroli, Bonomi, Faoro, Ferranti, Iriti, Picariello & Iametti, 2007).

Data were elaborated using the Lineweaver-Burk equation as follow:

$$1/F = 1/F_{max} + K_d/[ANS]^* F_{max}$$

Where F is maximum registered fluorescence intensity, [ANS] is the total fluorescent probe concentration (μM), F_{max} is the maximum fluorescence intensity (at saturating probe concentration) and K_d is the apparent dissociation constant of a supposedly monomolecular protein/ANS complex. F_{max} and K_d were calculated by standard linear regression fitting procedures. The ratio F_{max}/Kd, corrected for the protein content, gives the protein surface hydrophobicity (PSH) index.

Cysteine thiol accessibility measurements were performed according to Iametti, De Gregori, Vecchio & Bonomi (1996). A 0.1 ml aliquot of protein solution were added to 0.9 ml of 2 mM DTNB. After 15 minutes at room temperature, solutions were centrifuged for 10 min at *13200 x g*, and the absorbance of the supernatant was read at 412 nm.

Limited proteolysis and MALDI-TOF mass spectrometry

Limited proteolysis experiments were performed as follow: to 1 ml of β-conglycinin emulsion, were added 0.01 ml of trypsin solution (Sigma, TCPK treated, 10 mg/ml in 0.025 sodium acetate, pH 4.5) in order to reach a protease/substrate ratio of 1/100 w/w. Hydrolysis were carried out for 30 min at 37° C and stopped by addition of 0.02 ml of Soybean Kunitz Tripsin Inibitor (20 mg/ml in dd water). The hydrolyzed emulsion is then centrifuged in order to separate the fat phase from the aqueous one, and collected separately.

In order to extract the resulting peptides from each phases after the hydrolysis, the following protocol was applied: to one volume of each samples 3 volumes of exane and 2 volumes of acetone were added in a glass vial. After vigorous mixing and subsequently separation of the two immiscible phases, the upper phase (the apolar one, containing the hexane and the extracted lipids or polystyrene) was removed. This step was repeated three times. The acetone from the delipidized polar phase was removed by vacuum stripping, and then the samples were lyophilized.

The lyophilized samples were dissolved in dd water with 0.1 % TFA. After a cleaning passage, performed with C18 Zip Tip devices (Millipore) using the protocol provided by the manufacturer, samples were mixed either with the matrixes (α-cyano- 4-hydroxycinnamic acid or sinapinic acid) and loaded on the MALDI plates. MALDI-TOF mass spectrometry was carried out on a PerSeptive BioSystems Voyager DE-Pro spectrometer equipped with a N2 laser (λ 337 nm, 3-ns pulse width, 20-Hz repetition rate).

The instrument operated with an accelerating voltage of 20 kV. External mass calibration was performed with low-mass peptide standards including angiotensin I, (m/z ¼ 1296.68), bovine α^{s1}-casein 1-23 peptide (m/z ¼ 2764.55) and bovine insulin (m/z ¼ 5730.61). Mass spectra were acquired in the reflector mode using Delay Extraction (DE) technology, and analyzed by using the software provided by the manufacturer.

Results

Protein structural changes in solution and its reversibility

A first set of experiments was aimed at assessing the nature and extent of molecular modifications that the two proteins undergo when denatured in solution, in order to compare these changes with those occurring at oil/water interface. Denaturation was monitored through measurements of: 1) tryptophan fluorescence (providing information on the chemical environment of tryptophan sidechains); 2) far-UV CD spectroscopy (providing information on type and amount of secondary structure elements); 3) surface hydrophobicity (measuring the number and affinity towards suitable probes of hydrophobic patches on the protein surface (Cairoli *et al.*, 1994; Iametti *et al.*, 1993); 4) accessibility of cysteine thiols (that depends on their distance from the protein surface as a function of denaturation).

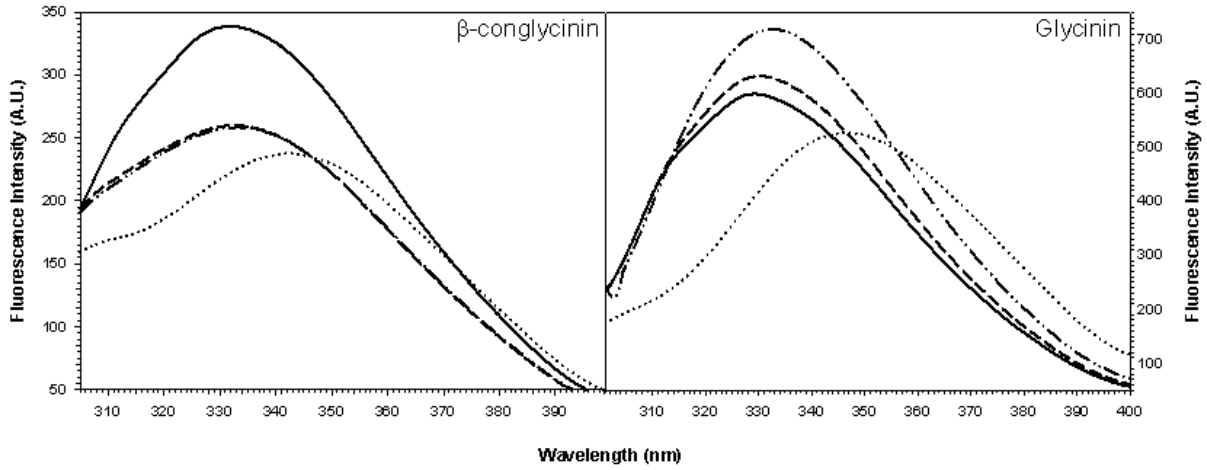

Fig 2.3: e*ffect of heat treatments on the emission fluorescence spectra of β-conglycinin and glycinin solutions (1 mg/ml). Shown are the average of at least three spectra taken on: untreated protein (—), urea denaturated protein (•••), protein heated at 75°C for 15 min (- - -), protein heated at 95°C for 15 min (–••–)*

Figure 2.3 presents the average fluorescence spectra for β–conglycinin and glycinin solutions before or after denaturation using temperature or chaotropes. Heating of protein solutions was carried out at 75°C and 95°C for 15 min, as those temperatures have been shown to be above the transition temperatures for β-conglycinin and glycinin by differential

scanning calorimetry. As shown in Figure. 2.3, heat treatment of β-conglycinin solutions does not shift the maximum emission wavelength of tryptophan fluorescence (336 nm) after heating at both 75° C and 95° C and subsequen t cooling. On the other hand, treatment with 8 M urea has been shown to unfold β-conglycinin completely (Sze *et al.*, 2007), and the tryptophan emission maximum is red shifted about 8 nm (344 nm). A red shift in the fluorescence emission maximum indicates transition of the tryptophan sidechain to a more polar environment. Heat-treated glycinin solutions show a very small red shift (330 nm vs. 329 nm in the non-heated protein) after heating at 75° C for 15 min, that increases to 4 nm (333 nm) heating at 95° C. T he higher shift that occurs in the 95° C treated sample means that glycinin treated at this temperature loses a higher amount of its structure with an exposure of its tryptophan-containing regions to the aqueous phase, compared with glycinin heated at 75° C. It is impor tant to note that differential scanning calorimetry reports transition temperatures higher than 85° C for glycinin, depending on the environmental conditions. Results in Figure 2.1 clearly demonstrate that glycinin's structural changes already occur at temperatures below 80° C. Upon complete unfolding of glycinin in 8 M urea a very large red shift is observed (18 nm).

Temperature-induced structural changes were also investigated by CD spectroscopy on solutions of the protein after heating for 15 min at 75° C and 95° C and subsequent cooling.

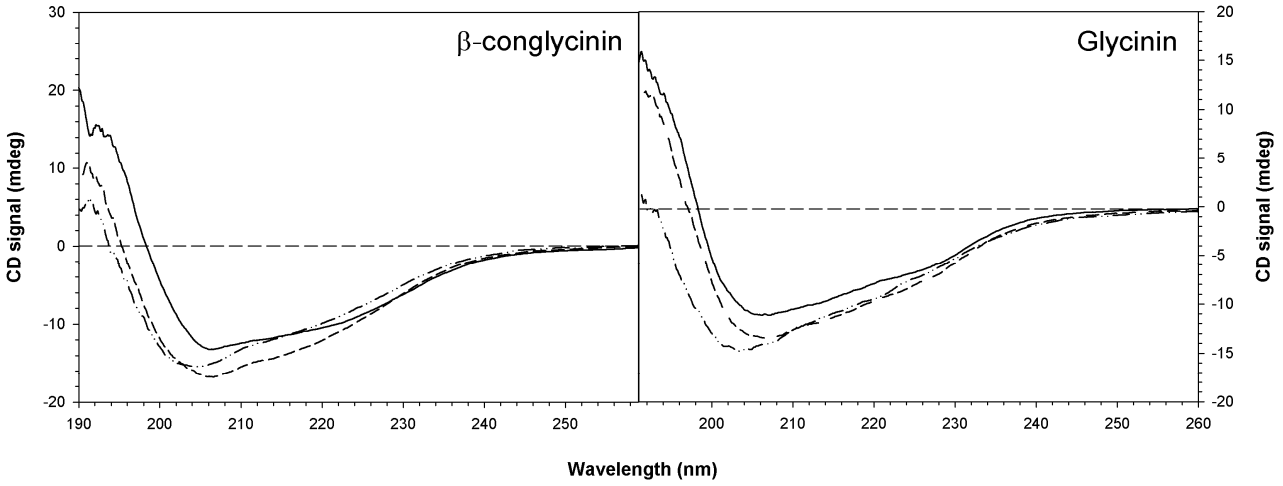

Fig 2.4: *Effect of heat treatments on the far-UV CD spectra of β-conglycinin and glycinin solutions (0.2 mg/ml). Shown are the average of three spectra taken on: untreated protein (—), protein heated at 75°C for 15 min (----), prote in heated at 95°C for 15 min (–••–).*

No changes were detected in as for Trp-dependent ellipticities in the near-UV CD region in the case of β-conglycinin, where urea gave a >60% decrease in signal intensity (not shown). A marked decrease in signal intensity was observed in the same spectral region for glycinin, confirming the indications from the fluorescence measurements. Exposure at either protein at temperatures above 75° C caused modest but irreversible temperature-induced changes in their secondary structure, as shown in Figure 2.4. Analysis of changes in secondary structure elements indicated that the alpha-helix content decreased from 13/14% to about 11% after treatment at 95° C, together with that of beta-sheet regions (from 5.5 to 4.5-4.6%), with a concomitant increase in random coil regions (from 33.8-33.2% to 36.7-36.9%). No residual features were evident from 260 nm to 200 nm when 8 M urea was added to either protein. The intense urea absorbance prevented recording spectra at shorter wavelengths. The overall surface hydrophobicity index (PSH), previously used for the identification of changes in the structure of proteins, defines the affinity of a hydrophobic probe for the protein (Cairoli *et al.*, 1994; Iametti *et al.*, 1996) was also mesured. Table 1 summarizes the data obtained from ANS hydrophobicity measurements on the protein solutions. The PSH of β-conglycinin solutions treated at 75° C is 2.3 times higher than that of the native protein, with an increase of both F_{max} and K_d values. The increase in fluorescence intensity at saturating concentrations of the probe (F_{max}) indicates an increment in overall surface hydrophobicity, that is to say that novel hydrophobic sites are generated. The concurrent increase in the apparent dissociation constant of the protein/probe complex (K_d) conversely indicates that the average affinity of surface hydrophobic sites for the probe is somewhat decreased. Also, no significant changes in cysteine thiol accessibility were detected for β-conglycinin heated at both 75° C and 95° C.

Therefore, it appears that β-conglycinin undergoes a supra-molecular rearrangement, likely involving a re-organization of its quaternary structure without affecting its tertiary structure. The occurrence of temperature-dependent aggregation in the proteins investigated here has been the subject of recent studies (Keerati-u-rai *et al.*, in press), that pointed out the higher sensitivity to thermal aggregation of glycinin with respect to β-conglycinin. The values of PSH for glycinin solutions double after treatment at 75° C, without a significant change in K_d, suggesting the formation of novel hydrophobic sites on the protein's surface, without affecting the overall affinity of these sites for ANS.

After heating at 95° C for 15 min, the value of PSH of glycinin solutions increases further (2.2 times with respect to 75° C), again with no significant changes in K_d. Cysteine

thiol accessibility changes in glycinin are apparent only after treatment at 95° C, where the number of thiols accessible to the specific reagent used here is twice that in the native and in the 75 °C treated protein. These data indicate that although structural changes already occur after heating at 75° C for 15 min glycinin un folds (irreversibly) and loses most of its tertiary structure at temperatures higher than 95° C.

SAMPLE	F_{max} (fluorescence intensity in arbitrary units)	K_d (μM)	P.S.H. (fluorescence intensity · mg^{-1} · μM^{-1})	-SH accessibility (A_{412})
β-conglycinin UH	76.34 ± 2.6	26.53 ± 3.21	**2.9**	**0.064 ± 0.016**
β-conglycinin 75°C	277.78 ± 12.3	41.56 ± 5.22	**6.7**	**0.077± 0.003**
β-conglycinin 95°C	312.5 ± 10.85	40.75 ± 4.07	**7.7**	**0.056±0.021**
Glycinin UH	104.17 ± 2.62	27.98 ± 2.06	**3.7**	**0.074±0.004**
Glycinin 75°C	178.57 ± 4.51	27.06 ± 2.21	**6.6**	**0.079±0.012**
Glycinin 95°C	476.19 ± 25.29	33.05 ± 4.96	**14.4**	**0.162±0.011**

Tab 2.1: *Protein surface hydrophobicity (PSH, assessed by titration with ANS as the hydrophobic probe) and accessibility of cysteine thiols (determined using the specific free thiols colorimetric reagent DTNB) in 1 mg/ml β-conglycinin and 1 mg/ml glycinin unheated and heated solution (75°C or 95°C for 15 min)*

Protein unfolding in emulsions

We first assessed whether the physical treatments involved in emulsion preparation had any effect *per se* on the structural features of β-conglycinin and glycinin. Protein solutions were passed through a Microfluidizer under the same conditions used for emulsion preparation, but in the absence of oil. No spectroscopically-detectable structural changes were detected on either glycinin or β-conglycinin after the dynamic pressure treatment. No changes in PSH occurred in the case of glycinin, whereas a small reduction of the number of hydrophobic surface patches was observed in the case of β-conglycinin, likely as a consequence of the aggregation phenomena detected by multi-angle static light scattering and reported and discussed in previous work (Keerati-u-rai & Corredig, 2009b).

As shown by the solid-state and solution fluorescence spectra in Figure 2.5, β-conglycinin undergoes a structural change at the oil/water interfaces. In particular, β-conglycinin tryptophans seem to increase their exposure to solvent water when the protein interacts with the oil surface. This is unexpected, as other proteins have shown to move

their tryptophans to a more hydrophobic environment (i.e. closer to the oil interface or inside the non-polar phase) when interacting with oil phase, so that their emission spectrum is blue-shifted with respect to that of the native protein (Castelain *et al.*, 1994).

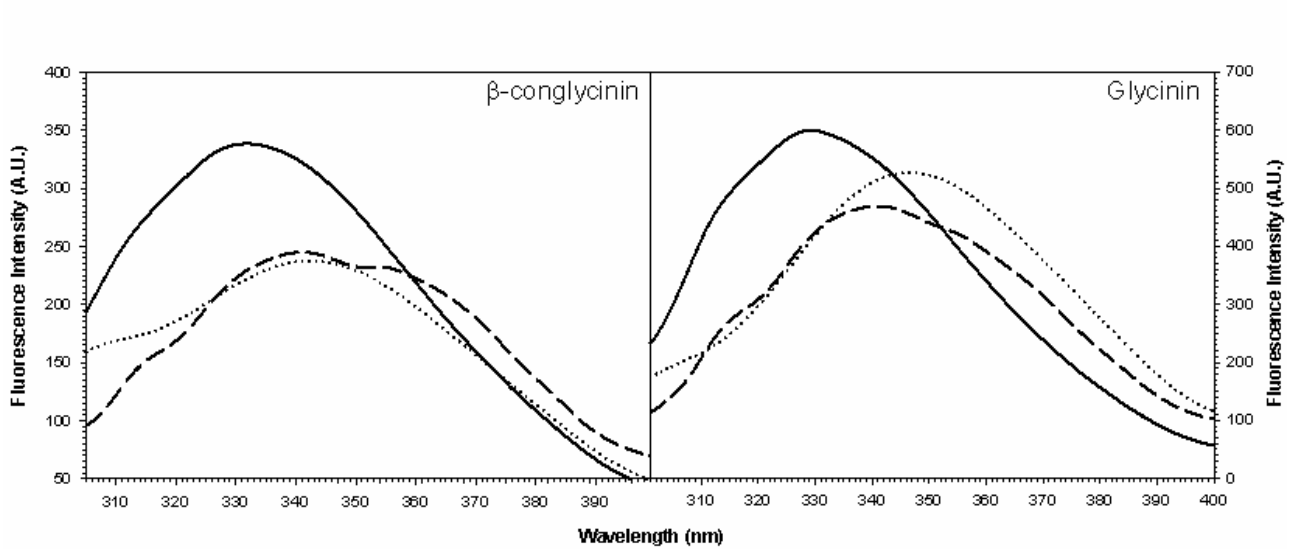

Fig 2.5: *Emission fluorescence spectra of 10% oil in water emulsions (resuspended washed cream) prepared with 10 mg/ml β-conglycinin or glycinin. The average of at least three spectra taken on (w/v) unheated emulsion (---) are compared with those of untreated 1 mg/ml protein solutions (—), and of proteins in 8 M urea (1 mg/ml)(•••).*

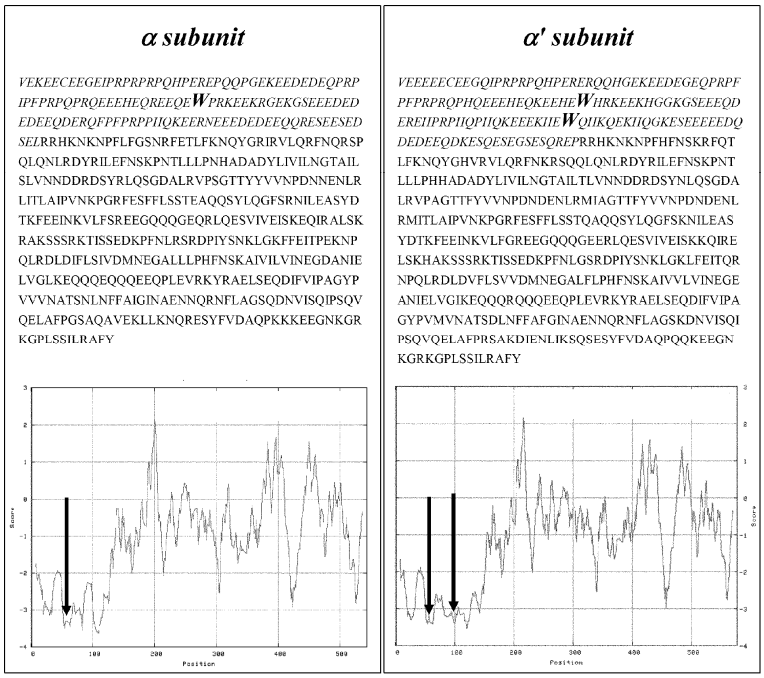

Fig 2.6: *Primary structure and Kyte and Doolittle hydrophobicity profiles of tryptophan containing subunits of β-conglycinin (α and α' subunits). Adapted from http://www.uniprot.org/uniprot/P13916 and http://www.uniprot.org/uniprot/P11827. In italics are indicate the extensions regions; arrows show the tryptophans positions into the Kyte and Doolittle hydrophobicity profiles of both subunits*

As shown in Figure 2.6, tryptophans in the mature form of β-conglycinin are present only in the in the N-terminal extension regions of the α–subunit (Trp_{63}) (http://www.uniprot.org/uniprot/P13916) and of the α' subunit (Trp_{63}, Trp_{100}) (http://www.uniprot.org/uniprot/P11827). Figure 2.6 also shows the Kyte and Doolittle hydrophobicity profiles (Kyte *et al.*, 1982) of α and α' subunits, indicating that in both proteins the tryptophan residues are present in their least hydrophobic areas. It is therefore possible to hypothesize that while the core region interacts with the oil phase, the extension regions of these subunits protrude in the aqueous medium.

Results from limited proteolysis experiments are shown in Figure 2.7. These experiments gave us other molecular details of the soy β-conglycinin adsorption behavior. After digestion with trypsin of the emulsion, some peptides were released into the aqueous phase (red highlighted) including the tryptophan containing regions in the extension domains, and other are retented into the fat-phase (green highlighted). Large peptides from the core region are released as well. These peptides come from the least hydrophobic regions of this domain.

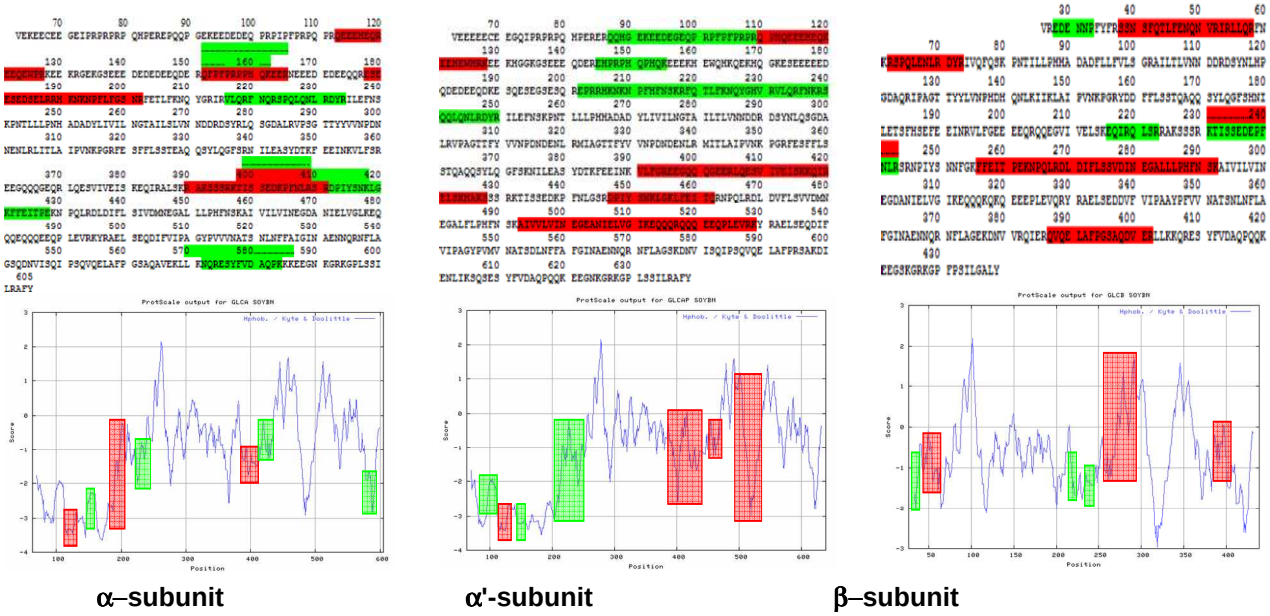

Fig 2.7: *Primary structure and Kyte and Doolittle hydrophobicity of α, α' and β conglycinin. The red-highlighted regions are peptide retained into the fat phase, whereas the green highlighted are peptide released into the aqueous phase*

These results confirm previous hypotheses put forward by Maruyama *et al*. (1999) and based on studies on recombinant subunits of β-conglycinin. However, it is important to note that the extension regions of α and α' subunits are not completely unfolded at the oil/water interface, because the protein spectrum is only red shifted 5 nm in emulsions, less than the 8 nm observed upon complete denaturation with 8 M (Figure 2.3). The fluorescence spectra of glycinin-based cream also show that the tryptophan residues are exposed to the aqueous media when interacting with the oil phase. In addition, glycinin at the interface is not completely unfolded, showing an 8 nm red shift when adsorbed to the lipid surface, whereas an 18 nm red shift was observed for the completely denatured protein in 8 M urea.

To determine if heating would cause additional structural changes to the proteins once at the interface, fluorescence spectra were also collected for emulsions after heating.

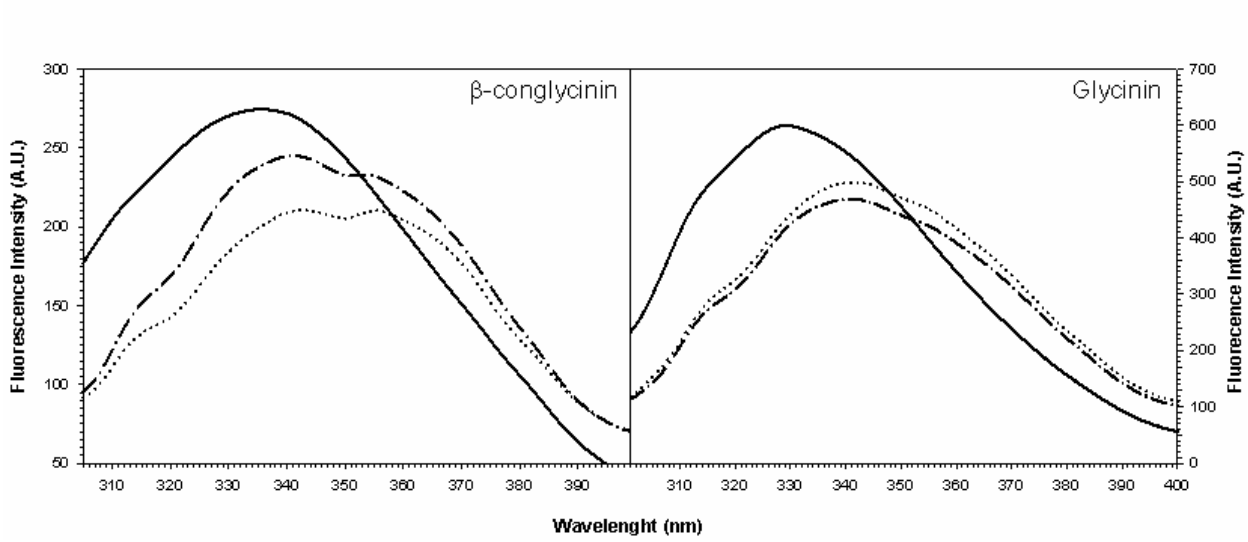

Fig 2.8: *Effect of heating on the emission fluorescence spectra of the 10% oil in water emulsions before heating or after heating (resuspended washed cream). The average of at least three spectra taken on unheated emulsions (–•–) is compared with that taken on an 1 mg/ml untreated protein solution (—) and the same emulsions heated at 75°C for 15 min (for β-conglycinin) and 95°C (for glycinin) (•••).*

As shown in Figure 2.8, heating a β-conglycinin emulsion at 75° C or a glycinin emulsion at 95° C does not affect the molecular st ructure of the proteins adsorbed onto the lipid droplet, as if the interactions ensuing from adsorption at the interface were strong enough to prevent further structural changes.

Conclusions

In this work structural changes of β-conglycinin and glycinin in solution were compared to those occurring when the proteins are adsorbed at the oil/water interface. Both proteins undergo structural modification after adsorption on the oil droplet surface. From the standpoint of protein chemistry, the modifications occurring at the interface with the proteins investigated here have some peculiar traits, in what both these proteins expose their tryptophan-containing extension regions to the aqueous phase rather than to the droplet interior, as observed for other proteins. It is very important to note that, in β-conglycinin, tryptophans are present in the extension domains of α and α' subunits, and the fluorescence data presented here confirm previous results (Maruyama *et al*., 2002)

demonstrating that the polar extension regions in these proteins are important for their emulsifying ability.

These results support the hypothesis that while the α and α' core domains interact with oil phase, the extension regions protrude into the aqueous phase and stabilize the emulsion droplets by providing the necessary polar regions. Also glycinin's tryptophans containing regions are exposed to the aqueous phase. However, the multiplicity of glycinin's genetic variants makes it much more challenging to derive definite answers from the hydrophobicity profiles of this protein, and some more detailed proteomic work is needed to better understand which portion of the protein anchors to the interface.

From the standpoint of applying this molecular information to food technology, it is also interesting to note that heat treatment does not affect the structural features of either protein once they are adsorbed at the oil/water interface. In other words, the modifications occurring upon adsorption at the interface appear to "lock" the protein structure in a conformation that is insensitive to further physical denaturation, at least under the temperature/time regimes employed in this work.

As a matter of fact, it is somewhat expected that, in emulsions, the structural regions more sensitive to the entropic changes ensuing from alteration in the water structure (i.e., the protein hydrophobic core) are at least partially buried into the non-polar lipid phase, and thus are insensitive to temperature-dependent changes in the colligative properties of the solvent.

The various peculiarities of these systems and their practical relevance seem worth further investigation. We are currently addressing the molecular details of the observed events, in an attempt to identify specific molecular determinants of the different behavior of these proteins, as well as the changes occurring during heating, and to assess whether the conformational changes reported here result in biologically relevant modifications when emulsions are consumed as food.

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Chapter 3)

ELUCIDATING THE STRUCTURAL CHANGES OF BOVINE β-LACTOGLOBULINE AFTER ADSORPTION ON HYDROPHOBIC NANOSTRUCTURED SURFACE

Introduction

The lipocalin family

Proteins belonging to the lipocalin family are extracellular and are united by the ability to bind and transport hydrophobic molecules of physiological importance. However, they have different biological functions and low sequence similarity (less than 25%). This family includes more than forty proteins, including: retinol binding protein (RBP, transports retinol), odorant binding protein (OBP), insecticyanin (INS, transporting chromophores), aphrodisin (carries pheromones), apolipoprotein D (APOD, transports sterols) and β-lactoglobulin (BLG, carries mainly fatty acids).

The main structure of the lipocalins, described for the first time for the RBP (Newcomer *et al*., 1984), consists of eight β-sheet (β-strand A-H) arranged in antiparallel position, and joined by a series of loops and connected from a portion of α-helix. The spatial organization of these structures creates a hydrophobic cavity in a sort of barrel (β-barrel) conserved in all members of the lipocalin protein, characterized by the ability to interact with hydrophobic molecules (Brownlow *et al*., 1997).

The major structural differences between proteins belonging to the lipocalin involve β-strands A, F, G and H (Flower *et al*., 1993). These considerations led us to hypothesize that the conserved regions in different lipocalin can play a key role in determining the folding of the hydrophobic core

β-lactoglobulin: general structural and functional overview

Beta-lactoglobulin (BLG) is a small protein (18,281.2 Da) present in the whey fraction of milk from many mammals, including cow, donkey, horse, sheep and goat, but it is absent in rodents and in human. In bovine milk whey it accounts for about 3 g/l, while the other whey proteins are much less abundant: 1 g/l of α-lactalbumin, 0.04 g/l of serum albumin (BSA) and 0.08 g/l of immunoglobulin (Bell & McKenzie, 1964).

β-lactoglobulin consists of nine β-sheet, of which eight are organized in the β-barrel tertiary structure characteristic of the lipocalins (Brownlow *et al*., 1997), and it has an α-helix located in the C-terminal tail (Figure 3.1).

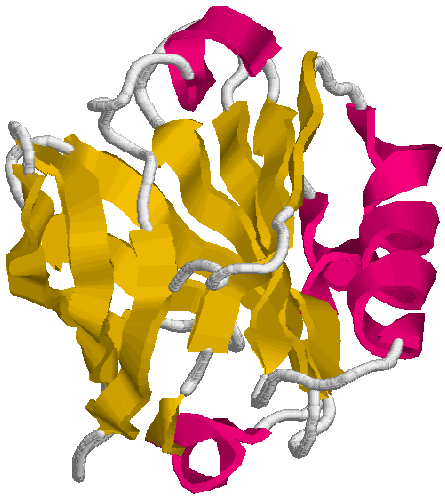

Fig 3.1: *3D structure of β-lactoglobulin monomer (pdb entry: 1UZ2)*

In the bovine milk BLG is mainly present in a dimeric form (about 36000 Da) and the dimer is in equilibrium with the monomer and other tetramer and octamer complexes. The dimer is created through non covalent interactions between the α-helix located in the C-terminus of the peptide chain, the ninth β-strand (I) and the AB loop of the two adjacent monomers (Figure 3.2).

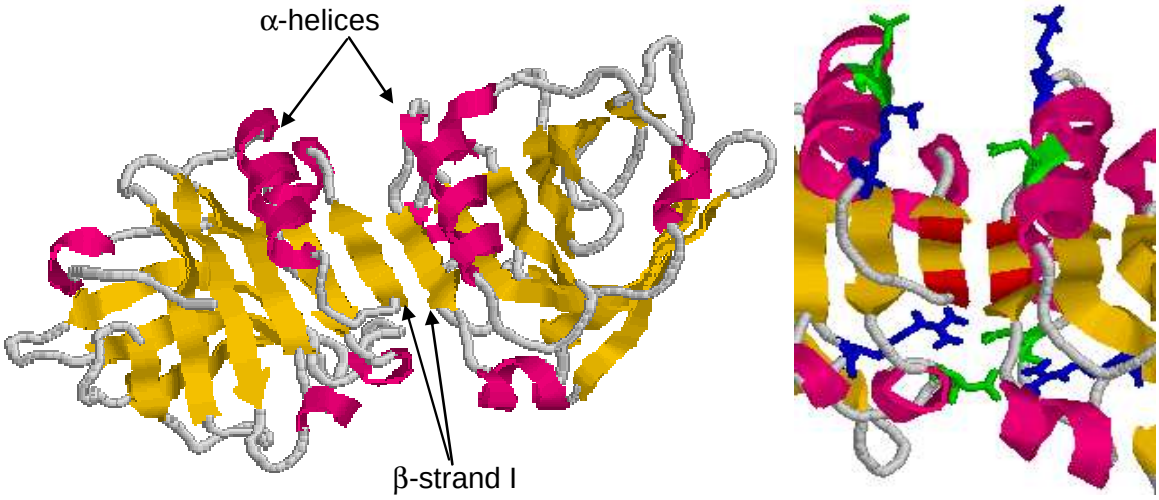

Fig 3.2: *β-lactoglobulin dimer in which are highlighted the portions of the molecule involved in the interaction. In the zoom on the right there is the detail of the interaction surface between the two monomers. Hydrophobic residues are in red, basic residues are in blue and green are the acids.*

The contact of the two β-strands I is stabilized by hydrophobic interactions and by four hydrogen bonds; Arg_{148} of the two adjacent monomers form two hydrogen bonds and the other two are created between Ser_{150} and His_{146} of one subunit with the respective amino acids on other. The hydrophobic interactions involve residues Ile_{147} and Leu_{149} of the two antiparaller β-strands, on the each monomer. Four ionic interactions are also involved in the stabilization of the dimer. The first two are generated between adjacent α-helices between Glu_{134} and Lys_{141} of each monomer present. The other portion of the molecule that stabilizes the dimer is the AB loop, residues Asp_{33} and Arg_{40} of two different monomers generate two ionic bonds (Brownlow *et al*. 1997; Sakurai *et al*, 2002; Lozinsky *et al*., 2006). At pH below 3, the ionic bonds are hindered due to the protonation of acidic residues and BLG is present as a monomer.

Each monomer is composed by 162 amino acids (34% of the residues are apolar) and contains five cysteine residues. Four of them are engaged in two disulfide bridges (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}). These covalent bonds stabilize the structure and the free thiol (Cys_{121}) in the native protein structure is hidden between the barrel and the α-helix. There are also two tryptophan residues, which are used as structural markers in fluorescence studies: Trp_{19} and Trp_{61} (Figure 3.3).

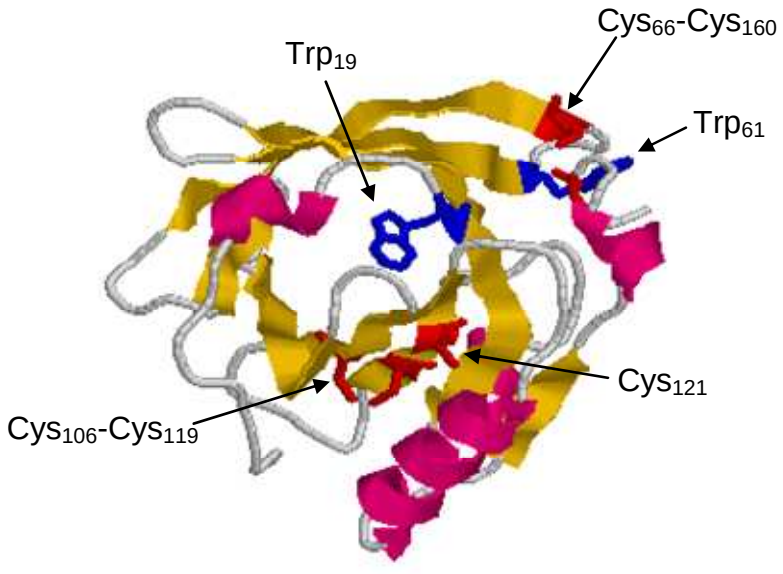

Fig 3.3: *3D structure of β-lactoglobulin monomer (pdb entry: 1UZ2) Cysteine residues are highlighted in red, tryptophans are highlighted in blue.*

Trp_{61} is located in the outermost position, near the Cys_{66}-Cys_{160} disulfide bridge. Instead, Trp19 is located at the base of the hydrophobic calyx, inside the protein structure and it is inaccessible to the solvent. This tryptophan is conserved in the lipocalin family. It may have a structural role in protein folding and it is believed that it is responsible for more than 70% of the fluorescence signal (Fessas *et al*., 2001).

There are several genetic variants of BLG (Godovac-Zimmermann *et al*., 1996), among them the most common forms are BLG-A and BLG-B. These differ in two amino acid residues, Asp_{64} and Val_{118} in BLG-A are replaced by Gly_{64} and Ala_{118} in the form B (Qin *et al*., 1999). The two genetic variants are characterized by a very similar isoelectric point. The pI for variant A is 5.26 while in the B variant the pI is 5.34 (McKenzie, 1971). The two genetic variants are also characterized by a different sensitivity to physical treatments, in particular to heat.

The replacement of Val_{118} with Ala, characterizing the B variant, results in minimal structural modifications that also determine conformational changes of the hydrophobic cavity, as the latter amino acid is located inside the structure and more precisely in the GH loop. These structural changes also affect the unfolding dynamics of the protein and are probably responsible for the lower thermal stability of BLG-B. The other residues involved in the differentiation of the genetic variants (Asp_{64}) is located in the EF loop and is exposed to solvent. Its replacement by glycine leads to conformational changes in BLG-B in the EF loop that explain the lower solubility and greater ability to polymerize and to gelatinize of the variant A than variant B. These structural changes also affect the disulfides exchange (Brownlow *et al*., 1997, Quin *et al*., 1999).

Despite a number of studies over the years, the function of BLG is not yet fully defined. Many authors have suggested a role for BLG transport of fatty acids and/or retinol, a function perfectly compatible with the barrel structure and its ability to bind hydrophobic molecules (Hambling *et al*. 1992; Perez & Calvo, 1995; Sawyer *et al*., 1998). In this regard, BLG may play a role in the transport of hydrophobic molecules through the digestive system because the protein resists to the proteolytic activity of the stomach. The hydrophobic molecules bind in the calyx of BLG β-barrel and remain protected unless the EF loop is open. This structural change occurs above pH 6.2 and is known as the “Tanfort transition”. The molecule in the hydrophobic cavity of the barrel, interacting with the protein through non-covalent bonds, is in equilibrium with the free form, in these conditions the equilibrium is shifted toward the bound form. If the interaction between protein and ligand is sufficiently strong, the BLG can carry a significant amount of ligand through the

stomach, reaching the small intestine where at basic pH BLG is hydrolyzed with the consequent release of the ligand.

Some authors have instead proposed a specific role of this protein in the digestion of fat globules (Pérez *et al*. 1991; Ragona *et al*., 2000), since the BLG increases the activity of pancreatic lipase, preventing their inactivation and promoting the removal of the fatty acid produced before they reach the duodenum. BLG also facilitates the transport of hydrophobic molecules in the gut in the case of infants. After milk digestion, the pH of the stomach is slightly acidic due to the immaturity of the stomach wall and the buffering capacity of milk (pH 5.5-6.5). However, this property is less clear in adults because the pH is more acid in the stomach and the ligand could be released before reaching the intestines.

Given the similarity with RBP, BLG may be involved in the transport of retinol, as confirmed by the identification of certain receptors in the gut for BLG (Papiz *et al*., 1986). Some authors (Brownlow *et al*., 1997), showed the presence of receptors for retinol-BLG complex in the intestine of calves, thereby confirming that the BLG could be involved in the transport of retinol from mother to infant by facilitating their absorption. It was also suggested that BLG could be the extracellular counterpart an intracellular protein able to bind and transport fatty acids.

The BLG isolated from bovine milk is present associated with different fatty acids and retinol, while in vitro is able to bind a large number of other amphiphilic and hydrophobic molecules, such as vitamin D, cholesterol, bilirubin (Zsila *et al.*, 2003), estradiol, progesterone and protoporphyrin IX (Tian *et al.*, 2006). The interaction of BLG with fatty acids, and in general, with the ligands, is hydrophobic in nature but unstable. Ligands interact with BLG mainly through the hydrophobic cavity of the barrel structure but some studies carried out using fluorescence hydrophobic probes show that there are other possible binding sites.

Narayan & Berliner (1998) have identified the presence of a binding site for fatty acids that is created between the hollow the α-helix and the main barrel. Dong *et al*. (2005) confirm the presence of a surface hydrophobic site located towards the N-terminal region of the peptide chain which may interact with hydrophobic molecules. The presence of two binding sites confirms the ability of BLG to interact simultaneously with two different molecules, such as retinol and palmitic acid (Narayan & Berliner, 1998). Numerous studies have been devoted to defining the nature of the naturally occurring ligands on BLG and 70% of BLG monomers after purification from bovine milk are associated with lipids (Perez

et al., 1989). Among them, palmitic, oleic and stearic acids are the compounds mainly related to the protein. The palmitic acid is a fact of course tied to the BLG molecules in greater quantities (Collini *et al*., 2003), the protein is also associated with retinol, lactose, that are compounds naturally present in milk.

β-lactoglobulin denaturation by heat treatment, salts and chaotropes

The heat treatment of milk, which is a process commonly used in the production of various dairy products, leads, in the case of proteins, some structural changes that often affect the quality of the finished product. The structural modifications induced by heat in whey proteins determine multiple effects, which can be modulated in order to improve the functional properties of proteins such as the foaming and emulsifying capacity (Moller & Jones, 1987; Konrad & Lieske, 1994).

The structural changes of BLG after heat treatment are related in the first place to the dissociation of the dimer and the formation of "activated" or "modified" monomers (Cairoli *et al*. 1994; Iametti *et al*. 1996; Prabakaran & Damodaran, 1997). Spectroscopic studies have shown that, at temperatures above 65-70° C, these changes become irreversible with the consequent formation of aggregates stabilized by disulfide bonds and by hydrophobic interactions (Iametti *et al*., 1996, Griffin *et al*., 1993, Hoffmann & Val Mil, 1997). In microcalorimetry studies, BLG showed two energy-independent domains with different thermal stability: the more thermostable domain appears to be that contain Trp_{19}, whereas the domain with a lower transition temperature is that formed by most of the α-helix region.

The events connected with the formation of these associated forms of BLG have been described by several authors (Parris *et al*., 1991, Griffin *et al*. 1993; Roefs & De Kruif, 1994; Iametti *et al*., 1995, 1996, 1998; Relkin, 1998) and they have identified a series of temperature-dependent sequential stages that may be summarized as follows:

Initialization phase:	$\mathbf{B_2 \leftrightarrow 2B \rightarrow B^{SH}}$	
Propagation phase:	$\mathbf{B + B^{SH}_i \rightarrow B^{SH}_{i+1}}$	$\mathbf{_i \geq 1}$
Termination phase:	$\mathbf{B^{SH}_i + B^{SH}_j \rightarrow B_{i+j}}$	$\mathbf{_{i,\,j} \geq 1}$

The initiation stage is represented by a number of reversible reactions, in which the BLG dimer (B_2) dissociates into two monomers (2B). The dissociation, favored by mild heating, induces a structural rearrangement, thus changes the spatial relationships

between in the surrounding of hydrophobic residues, but not in the chemical environment of Trp_{19} and Trp_{61} (Iametti *et al*., 1996). These reversible reactions are followed by an irreversible reaction in which the BLG exposes its free thiol group (Cys_{121}) and an adhesive hydrophobic surface, both previously hidden at the monomer-monomer interface. The now reactive protein (B^{SH}) can actually starts the associative process. At the propagation stage, the BLG, reactive thiol group reacts via a thiol disulfide exchange reaction with the Cys_{66}-Cys_{160} disulfide bond on the outer surface of an unmodified BLG molecule. It forms an intermolecular disulfide bond and a new reactive thiol group becomes accessible. The stage of propagation can then be repeated several times, thus leading to the formation of linear aggregates. The polymerization process stops with the termination stage, when two reaction intermediates react with each other, forming a polymer without reactive thiol groups.

In this model, the monomers are linear, but the polymer aggregates may also assume a spherical form (Hoffmann *et al*. 1997). Other studies have shown that non-covalent interactions also play an important role in the aggregation (McSweeney *et al*., 1994a; McSweeney *et al*. 1994b, Qi *et al*. 1995; Schokker *et al*., 1999, Bauer *et al*., 2000). In particular, some authors (Iametti *et al*. 1995; Galani *et al*., 1999) have highlighted the predominant role of hydrophobic interactions in defining the irreversible changes induced by heat treatment in areas of temperature between 75 and 90 °C.

Even the size of the aggregates is correlated with the temperature treatment. At temperatures around 65 °C formation of small oligo mers may occours, including trimers and pentamers (Bauer *et al*., 1998). Prolonging the treatment time there is a significant decrease of these species, simultaneous with the appearance of large aggregates. The same Authors (Bauer *et al*., 2000) have suggested a mechanism of aggregation where there is formation of linear aggregates and / or branched structure.

The tendency to form aggregates is also affected by pH, since the reactivity and accessibility of the thiol group depend on it. Between pH 7.0 and pH 8.2, the protein undergoes the Tanford transition; this leads to an increase of reactivity of the -SH, which determines the pH-dependent physical and chemical properties of BLG. At pH 8.0, the thiol group is more readily available for reactions, and there is an increase of the disulfide exchange rate, while at lowest values, below pH 7.0, the molecule must be heated or unfolded in some other way, to expose their thiol (McSweeney *et al*., 1994a; McSweeney *et al*. 1994b, Hoffmann and van Mil, 1997).

A high acidic pH values (2.0-3.0), BLG has only positive charges, so the aggregation mechanism is different: the exchange of disulfides hard to come by, because the thiol groups are very stable at low pH where they are present in the protonated form, and there is a repulsion of electrostatic nature between the monomers, even though the electrostatic effect may be shielded by the addition of salts.

Another factor correlated with the thermal aggregation of BLG is the protein concentration. At physiological pH and after heat treatment (Iametti *et al*., 1995), soluble BLG aggregates are formed which are stabilized by covalent and hydrophobic interaction, (Iametti *et al*. 1996; Prabakaran & Damodaran, 1997; Relkin 1998) whose size is concentration-dependent (Iametti *et al*. 1996; Galani *et al.*, 1997). A study conducted through the use of different techniques, has proposed that the unfolding of the protein is independent of concentration, whereas the associative phenomenon is heavily dependent (Iametti *et al*., 1995).

Taking into account all the variables described above for the formation of aggregates, Verheul, Roefs and de Kruif (1998) have extended this forming mechanism (Roefs and de Kruif, 1994) to describe the denaturation / aggregation of BLG over a wide range of conditions (pH, temperature and ionic strength). According to this model, the aggregation induced by heat can be interpreted through a reaction scheme consists of two phases: denaturation, followed by the reaction of aggregation. This second stage would start at a critical concentration of primary particles formed in the first and would not be limited to exchange reactions between-SH and-SS-.

The structuring/destructuring action of a salt depends on its ability to modify the hydrophobic and electrostatic interactions involved in defining the protein structure. Information on the characteristics of the different ions are given by the Hofmeister series, where the different salts are classified according to their lyotropicity. Among the anions, in descending order of lyotropicity, we find: sulfate, phosphate, chloride, bromide, thiocyanate and perchlorate. Lipophilic ions such as phosphate or sulfate (polyvalent anions with highly distributed charges) have a positive effect on protein stability and for this reason they are normally used as salts in the preparation of buffers for the proteins or for protein stabilization in industrial preparations. At the opposite end of the Hofmeister series there are ions that are monovalent and larger such as thiocyanate and trichloroacetate. They exhibit modest interactions with the solvent (lipotropic) and, due to their poor solvation, they are able to penetrate into the protein structure resulting in destabilizing action towards ionic pairs in the protein structure.

Differential scanning calorimetry (DSC) studies (McPhail & Holt, 1999) have identified major differences in the rate of aggregation and denaturation of BLG samples dissolved in buffers containing anions of different types. The primary role of the ionic strength or chaotropic thiocyanate as the solutions of BLG is to dissociate the dimeric form to give monomers characterized by a structural organization similar to that of native or modified protein. The degree of these changes, as evidenced by several authors, affects the molecular aggregative properties.

Some authors have shown that heat treatment of BLG in the presence of potassium thiocyanate (KSCN) at non denaturating concentrations produces polymeric species with molecular properties different from those obtained by simple heating of the protein (Iametti *et al*., 2001). In these works it was determined that the effects of thiocyanate involves, in addition to the expected dissociation of the dimer, the structural changes dependent on salt concentration, that affect the subsequent formation of aggregates induced by heat treatment.

By studying the effects of heating BLG in the absence or presence of various concentrations of KSCN, it was possible to see that the dissociation of native dimer can be obtained already at a salt concentration of 0.2 M: heating the protein in contact with low concentrations thiocyanate yields modified monomers that have a tendency to form polymeric species mainly stabilized by non-covalent interactions. At higher concentrations of thiocyanate (1 M), at pH 6.8, the protein thermal stability is reduced because salt can probably access its internal areas, altering the electrostatic interactions at sites normally hidden to the solvent. The salt procures structural changes such as an increase in exposure and reactivity of the thiol group of Cys_{121} and of disulfide groups. This effect resulting from heat treatments, favors the formation of covalently stabilized dimers, whose formation is dependent on the concentration of salt and protein. Even higher thiocyanate concentrations (2 M) the structure of the monomer is altered and its sensitivity to heat, thus making possible the formation of insoluble macroaggregates.

Hydrophobic surfaces as unfolding agent

Proteins adsorb onto almost all surfaces, whether air/water, oil/water, or solid/water. Proteins are surface active, which implies that they lower the interfacial free energy upon adsorption. For instance, protein adsorption at an air–water interface lowers surface tension by about $30 mN*m^{-1}$, which equals 0.03 $J*m^{-2}$. There is only one exception: the adsorbent is a solid that is hydrophilic and charged, and the protein has a charge of the

same sign as the solid and is a ‘‘hard’’ protein. The latter implies that the protein has a relatively stable globular conformation, i.e., a fairly high $D_{N\rightarrow U}G$. ‘‘Soft’’ proteins also adsorb at hydrophilic solid surfaces, even of the same charge. Adsorption may thus primarily involve electrostatic attraction, in which case protein conformation is not greatly affected. However, other solids, oil, and air provide hydrophobic surfaces, where the main driving force for adsorption generally is hydrophobic interaction. Since most apolar residues are buried in the core of a globular protein, adsorption generally involves a marked change in conformation. This is made evident by results of spectroscopic studies, which show a change in secondary and loss of tertiary structure in adsorbed protein. DSC applied to an adsorbed protein generally shows a denaturation peak that is smaller (or even negligible), and that occurs at a lower temperature as compared to the protein in solution (Corredig *et al.*, 1995).

Adsorption of enzymes generally leads to loss of enzyme activity, whether measured in the adsorbed state or after desorption. By and large, the activity loss is greater under conditions (temperature, pH, etc.) where conformational stability is minimal, and if the adsorbent is more hydrophobic, presumably because the driving force for conformational change is greater. It is sometimes observed that adsorption from a more dilute enzyme solution leads to more inactivation. The explanation may be that at low concentration adsorption is slow, allowing adsorbed molecules to expand laterally, which implies conformational change. If adsorption is fast, a densely packed adsorbed layer is rapidly formed, which would prevent lateral expansion. In agreement with this, it has been observed that some proteins do not greatly change conformation when merely adsorbing onto an air/water interface, but when the air/water surface is expanded, for instance by deforming an air bubble, considerable change occurs (Noskov, 2009). Beating air into a protein solution can therefore cause denaturation. It has further been observed for several enzymes that adsorption onto an oil/water interface causes complete inactivation, whereas only partial inactivation may occur due to adsorption onto an air/water surface. The reason may be that hydrophobic segments of the molecule can penetrate into an oil phase, but not into air. This would be because the net attractive energy between these segments and oil can be greater than that between segments and water, whereas the attractive energy between any group of a protein and air will be virtually negligible.

This must cause a greater driving force for loss of native configuration at the oil/water interface. A fairly stable enzyme like lysozyme, which can regain activity after various unfolding treatments at low temperature, does not regain it after adsorption onto oil

droplets, even at its isoelectric pH. This leads to the important conclusion that more than one unfolded state can exist, and that some of these states permit return to the native state, whereas others do not.

The polypeptide backbone is fairly polar, but several side groups are hydrophobic. All proteins are surface active and adsorb at O/W and A/W surfaces. Globular proteins often retain a fairly compact form, although conformational changes do occur (Figure 3.4).

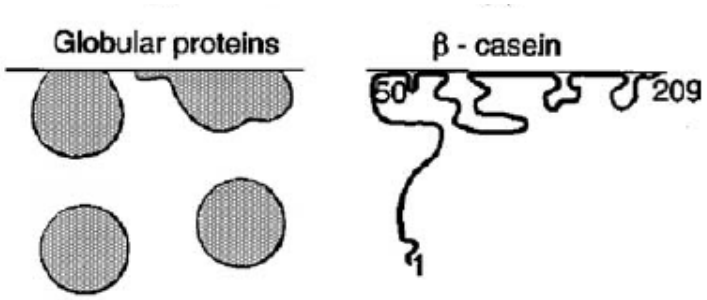

Fig 3.4: *Very approximate schematization of adsorption mode of various polymers from an aqueous solution*. *From Waalstra P., Phisical Chemistry of Food*

Non globular proteins and intrinsically disordered, such as gelatins and caseins, tend to adsorb in various way. β-casein average conformation on adsorption is fairly well known. The surface activity of a protein and an amphiphile (SDS) are compared in Figure 3.5.

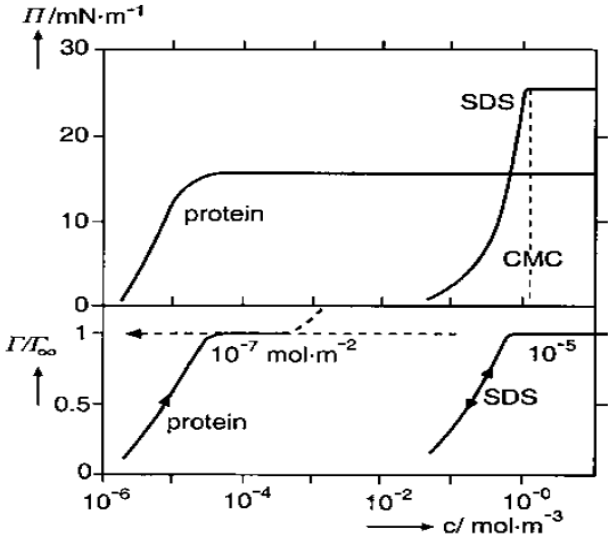

Fig 3.5: *Surface pressure (Π) and surface excess (Γ; the plateau value Γ^{∞} is indicated near the curves) at the triglyceride oil–water interface as a function of the concentration in solution (c) for a protein (b-casein) and an amphiphile (SDS). From Waalstra, Phisical Chemistry of Food*

It is seen that the protein is much more surface active. The molar bulk concentration needed to reach Γ^{∞} differs by 4 orders of magnitude. If the mass concentration is plotted, the curves are closer, but the difference is still by more than two orders of magnitude. The main cause is the larger molar mass of the protein. It implies that the free energy of adsorption per molecule (roughly equal to Π/Γ N_{Av}) is very much larger than that of the amphiphile; for the protein it would be about 60 times k_BT, and for the amphiphile of the order of 1 k_BT. Consequently, the affinity of the protein for the surface is far greater than that of the amphiphile.

On the other hand, the value of P reached is clearly larger for the amphiphile, provided that the surfactant concentration is high enough. The explanation must be that for a polymer, and hence a protein, a very dense packing of surfactant material at the interface cannot be reached. This is in accordance with the observation that for most polymers and for most amphiphiles the surface excesses expressed in unit mass are roughly the same: a few mg/m^{-2}, despite the ‘‘thickness’’ of the polymer layer being clearly higher for a polymer (10nm) than for an amphiphile (about 2.5 nm).

For some proteins at some conditions, multilayer adsorption can occur, as indicated by the dotted curve for Γ/Γ^{∞}; a second layer is very weakly adsorbed. Apart from this phenomenon, high surface loads can be obtained by adsorption of protein aggregates (e.g., casein micelles) rather than free molecules; by formation of a gel layer at the interface (e.g., of gelatin at low temperature) or by covalent intermolecular cross-linking (e.g., formation of –S-S- bonds between β-lactoglobulin molecules). Generally, lateral interaction forces act between globular protein molecules in an adsorption layer, and these forces may strengthen markedly with time.

β-lactoglobulin denaturation by surface effect

The surface properties of β-lactoglobulin solutions has been the subject of numerous studies, but the conclusions of different authors on the degree of protein unfolding were not always the same. A work of Cornec *at al.* (2002), shows using C^{14} labeled protein that the area per mol. of an adsorbed β-lactoglobulin during the dynamics of adsorption was smaller than that for spread monolayer since β-lactoglobulin was not fully unfolded during adsorption. Wu *et al.* (2008) demonstrate a rapid initial unfolding followed by much slower rates at longer times after a time lag, with this time lag being shorter at lower surface concentrations. The effects of β-lactoglobulin surface concentration, pH, and ionic strength on the unfolding kinetics indicated that protein-protein interactions on nanoparticle

surfaces are important. The variation of the surface concentration of β-lactoglobulin as well as the presence of TFE did not influence the secondary conformational change on the surface. DTT, however, was found to result in a decrease in the β-sheet fraction with a corresponding increase in the random coil fraction.

Also in a work of Fang *at al.* (1997) a time dependent relation between time of adsorption and the protein structure changes were demonstred. These are also pH-dependent, which may partly reflect the quaternary structure of the protein. Adsorption generally appears to alter, but not destroy, the β-structure contained in the protein, and certainly at pH 7, the α-helix remains intact. There is by no means a complete randomization of the secondary structure of the protein. On the other hand, heating causes more profound changes, with extensive change and loss of β-sheet but with appearance of structures associated with aggregation of the protein. The denaturation by heat leads to a much less structured molecule than does adsorption. When BLG was adsorbed on the oil–water interface, the denaturation started with the loss of the exposed section of β-sheet structure, which may have a high probability for adsorbing first to the interface, after which a slow unfolding of the protein continued during the storage of the emulsion to give a more unordered structure.

Oil-in-Water emulsion as adsorbent

Over the past three decades, studies on emulsions were very numerous, but actually there is no a general theory that can describe in deep the formation process. Many theories have been proposed from time but only applied to specific cases under study.

An emulsion is defined as a system consisting of a mixture of two immiscible phases: the dispersed phase, which is at globular state, and the continuous phase which is the dispersion medium. The emulsions are part of a more general class of systems called biphasic colloids. Although these terms are often used interchangeably, the emulsions are characterized by the fact that both phases constituents of the system are liquid.

Examples of emulsions are present in many fields such as cosmetics, medical, photographic, and especially in the food. Many products are in fact stabilized by the presence of an emulsion which ensures a high shelf life. The best known examples are ice cream, creams, cakes, soups and many baked goods.

As mentioned above, the emulsion consists of two phases, one dispersed and one continuous interacting each other via an interface. The emulsions also tend to take an opalescent and whitish appearance account of the phenomena of light scattering at the

interface. This phenomenon is also related to the concentration of the dispersed particles and their size.

The emulsion is a highly unstable system that is not formed spontaneously. It needs the use of systems capable to generate energy which allows to mix, stir and then mix the two phases. In addition, the energy supplied to the system is essential to bring the molecule in contact with the surfactant phase, and if one use protein as stabilizing agents, sufficient energy must be provided to deform the protein structure and to facilitate adsorption.

In relation to the content of the aqueous phase and non aqueous phase it is should be possible to talking about water in oil emulsion if the second is less or *vice versa*, of oil in water if it is the non-aqueous phase is present in higher concentration.

A key role during the process leading to the formation of the emulsion is played by emulsifier substances. An emulsifier is a substance capable to act as a surfactant to stabilize an emulsion, i.e. by decreasing the surface tension of the liquids and then to facilitate the “wetting” of the interface. Emulsifiers are widely used as food additives. Examples of food emulsifiers are lecithin contained in egg yolk or derived from soy beans. A wide variety of emulsifiers are also used in pharmacy for the preparation of emulsions in the form of creams and lotions.

Polystyrene nanoparticles as adsorbent

Many different adsorbents are used to study protein adsorption. These adsorbents vary in charge, hydrophobicity and by the presence of specific groups on their surface. One of the most used liquid/solid matrix is polystyrene in micro or nano form. Virtually all proteins present in the system are adsorbed on the surface of the polystyrene nanospheres and virtually no free protein remains present in solution. This situation is advantageous for spectroscopic studies as all spectroscopic signals observed originate from adsorbed protein molecules and no contributions from free protein molecules need to be taken into account.

The large surface area per mass of polystyrene nanospheres and therefore the high binding capacity allows the use of low nanosphere concentrations. As a result, light scattering and light absorption can be minimized, which makes the use of spectroscopic methods like stopped-flow fluorescence feasible.

Fluorescence spectroscopy

The intrinsic fluorescence of protein molecules is very sensitive to changes in their tertiary structure. It informs about the local environment and dynamics of the fluorescent amino acid residues Trp, Tyr and Phe and of fluorescent cofactors like flavin, if present (Lakowicz, 1999). The position of the spectral maximum in the fluorescence spectrum, the fluorescence quantum yield, fluorescence polarization/anisotropy and the fluorescence lifetime are indicators for changes in the local environment of these fluorescent groups in the protein molecule. Fluorescence spectroscopy is not necessarily restricted to the fluorescence of intrinsic fluorophores. Specific residues on the protein can be labeled with a fluorescent group, and yield information about the environment of that group (Tan and Martic, 1990). In addition, fluorescent probes like ANS and thioflavin T inform about the structure of proteins, without the need for covalent interaction between the fluorescent probes and the protein (Karlsson, *et al*., 2000).

Analogous to CD spectroscopy, fluorescence spectroscopy can be used in combination with a system in which suspended adsorbent particles provide an interface for protein adsorption (Clark, *et al*., 1994; Tan, *et al*., 1990). In contrast to CD, the wavelengths used are higher and the concentrations of proteins and particles used are lower. This drastically reduces the effect of light scattering in fluorescence spectroscopy compared to CD spectroscopy. In addition, fluorescence spectroscopy is a more sensitive technique than CD spectroscopy. Besides steady state fluorescence spectroscopy, also time-resolved fluorescence spectroscopy (Czeslik, 2001; Czeslik and Winter, 2001; Maste, *et al*., 1996), stopped-flow fluorescence, and fluorescence anisotropy (Maste, *et al*., 1996; Pap, *et al*., 1996;Tan *et al*., 1990) have been used to investigate conformational changes of adsorbed protein molecules. Fluorescence anisotropy is directly related to the rotational correlation time of the fluorescent group involved (Lakowicz, 1999) and can be used to detect whether protein molecules are adsorbed. Adsorption of a protein molecule on a particle that is much larger than the protein itself increases the rotational correlation time of the adsorbed protein and thus increases its fluorescence anisotropy.

Limited proteolysis as technique to check protein unfolding

Detailed information on the conformation of caseins at the oil/water interface have been obtained using proteases by different authors (ref). By comparing the peptides produced during the proteolysis of the protein in solution or adsorbed at the interface, it has been

possible to identify the proteinase-sensitive bonds which are masked by being adsorbed to the oil phase.

Proteolytic digestion and antibody binding have shown that various regions of the αs^1-casein molecule are associated with the oil-phase in such emulsions and are effectively shielded from the action of water-soluble reactants. Recently it was studied (ref) the kinetics of the trypsin-catalyzed hydrolysis casein in solution and adsorbed to a soya oil-water interface and found that the phosphate-rich hydrophilic N-terminal end of the molecule appears to be less closely associated with the oil phase than the rest of the molecule. Trypsin-catalyzed hydrolysis of this protein in the emulsified system is a relatively ordered event initiated by cleavage of trypsin-sensitive bonds in this region. As with casein, the accessibility of certain of the arginine and lysine residues varied between the soluble and emulsified β-casein. These studies allowed definition of possible conformations for adsorbed casein.

Aim of this work

The aim of this work is to understand the structural changes that bovine β-lactoglobulin undergoes after adsorption on hydrophobic nanostructurated surfaces, in our case 46 polystyrene nanoparticles and oil in water nanoemulsion.

Material and methods

Chemicals

All reagents used were in the highest degree of purity commercially available, and purchased from Sigma Aldrich unless other provider are indicated. Nanoparticles (average size: 46 nm) were provided by Kisker Bioteck

β-lactoglobulin purification

To avoid the presence of species that characterize the partially denatured β-lactoglobulin commercial preparations we proceeded to purify the protein directly from fresh milk whey. The purification was performed by ion exchange chromatography of the serum obtained by skimming and subsequent removal of the casein fraction.

Whole milk was skimmed by centrifugation at 5000 *x g* for 30 min at 4° C. Skimmed milk was then filtered on gauze to remove any remaining cream. The casein fraction was removed exploiting isoelectric precipitation by acidification to pH 4.5 with HCl 5 M. The precipitated casein was removed by centrifugation at 7000 *x g* for 5 min at 30° C. The serum obtained was then neutralized to pH 7.2 by addition of TRIS base powder. To permanently delete the remaining portion of casein, whey was centrifuged at 11000 *x g* for 60 min at 20° C.

The separation of BLG from other whey protein was obtained by ion exchange chromatography. An appropriate volume of serum was diluted with water in 1:1 ratio to reduce the ionic strength and then loaded onto a DEAE-cellulose (Whatman) packed column previously equilibrated in 0.05 M Tris-HCl, pH 7.2.

BLG and many other serum proteins, including α-lactalbumin (ALA), are negatively charged and bind to the resin, while other proteins with different charge (lactoferrin, lactoperoxidase, lysozyme, etc.) are eluted in the unbound fraction. BLG was eluted from the resin using a step gradient of NaCl. The first step consists of 0.05 M Tris-HCl, pH 7.2, containing 0.1 M NaCl in which the α-LA is eluted, followed by a second step in which the BLG is eluted using a buffer containing 0.3 M NaCl. The purified BLG was then concentrated using an Amicon ultrafiltration device (Millipore) with a membrane of 10 kDa cut-off and subsequently lyophilized.

Protein concentration determination

The protein concentration of the different samples was determined spectrophotometrically by measuring the absorbance at 280 nm, using a 1 cm quartz cuvette in a Perkin Elmer Lambda 2S spectrophotometer. The concentration in mg/mL was calculated using an extinction coefficient, ε^{280}, equivalent to 0.93 mg $ml^{-1}*cm^{-1}$.

Emulsion preparation

Emulsions were prepared as follows: 0.5 g of soy oil (Sigma Co., St Louis, MO) were pre-emulsified with 9.5 ml of β-lactoglobulin solution (5 mg/ml in 0.05 M sodium phosphate buffer, pH 7) using a Vortex dispersing unit for approximately 1 min. The pre-emulsion was immediately homogenized in ice by using an ultrasound sonicator (MSE Soniprep 150) in 5 sonication cycles (1 minutes each) with a 14 microns amplitude intensity.

Emulsion characterization

Size determination

Emulsion droplets size were measured using a Mastersizer instrument (Malvern Instrument ltd.) equipped with Hydro SM manual small volume sample dispersion unit.

Determination of adsorption ratio

The emulsion was centrifuged at 13200 *x g* for 20 minutes to separate the lipid phase "cream" from the aqueous phase "serum". The “serum” was than harvested using a siringe. The cream thus obtained was weighed, washed and resuspended in an volume of buffer (0.05 M sodium phosphate, pH 7.0) to reach 5% content of oil.

The amount of protein adsorbed was determined by SDS-PAGE. To 0.05 ml of both “cream” and “serum” samples were added 0.05 ml of Laemmli denaturation buffer. The mix was boiled for 5 minutes and appropriate aliquots were then loaded on a 12% acrylamide gel. In order to construct a calibration curve, samples containing increasing amount of protein were loaded on the same gel too. After the electrophoretic run the gel was stained with Coomassie Blue. The protein amount present in the “cream” and “serum” phases were estimated by densitometry using a specific software (Image Master 1D, GE Healtcare)

Polystyrene nanoparticles/β-lactoglobulin system

Adsorption isotherms

In order to study the protein behavior on the NP interface it was necessary to determine the ideal protein concentration in relation to the available area. It is important that the experiment must not be either in conditions of excess or deficiency of the protein. A high amount of protein would lead to a supersaturation and consequently the spectroscopic signals may also be dominated by those arising from the native protein. Conversely, if the surface of the NP is not completely covered spectroscopic signals are too low and difficult to interpret.

The study of adsorption isotherms allowed the calculation of the protein amount necessary for the formation of a monolayer on the NP surface. For this purpose it was been prepared solutions with increasing concentrations of BLG (0.01 to 0.08 mg/mL) at constant concentration of NP (0.025 mL of original suspension/mL) in 0.05 M sodium phosphate buffer, pH 7. This was followed by an incubation of 60 minutes, in mild stirring conditions at room temperature, to promote interactions between the protein and the NPs. The adsorption isotherms were determined by measuring the concentration of the protein in the permeate after separation of NP/protein complex from the free BLG by ultrafiltration (Microcon devices, cut-off 50,000 Da). Protein concentration was determined spectroscopically by measuring the permeate absorbance at 280 nm

Determination of–SH accessibility

Determination of free –SH accessible groups in the different protein samples was performed by titration with the reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman, 1959) in order to evaluate the different accessibility in the free protein and in the protein present at the interface with nanoparticles. The interaction between DTNB and SH-group of a protein involves the formation of a yellow anionic species with a molar absorbance equal to 13600 $M^{-1}cm^{-1}$ at 412 nm.

In practice time dependent absorbance assay (λ: 412 nm, time: 15 min) were recorded at different temperatures (25° C, 45° C, 70° C) after addition to 0.95 ml of a NP-protein suspension (protein concentration 0.05 mg/ml) of 0.05 ml of 0.1 M DTNB solution in a 1 ml quartz cuvette. Two tests were also performed with the protein without NP (same concentration: 0.05 mg/ml, same temperatures) and only 0.05 M sodium phosphate buffer, pH 7.0.

Evaluation of protein surface hydrophobicity: titration with ANS

Many studies have pointed out that the only tryptophan fluorescence is not sufficient to provide all the information on structural changes affecting the hydrophobic regions of proteins, when they change their properties of surface hydrophobicity (Eynard *et al*. 1992; Iametti *et al* ., 1998).

Changes in surface hydrophobicity properties of proteins can be measured spectrofluorimetry, by using an appropriate hydrophobic markers, such as 1-anilino-8-naphthalene sulfonate (ANS), fluorophores able to interact with hydrophobic sites on the proteins surface.

In this regard, fluorescence measurements have been conducted on protein/NP complexes in order to assess any change in protein surface hydrophobicity. Tests were performed on solutions containing increasing amounts of ANS (0 to 0.2 mM). The fluorescence values, expressed in arbitrary units (AU), were then analyzed as a function of total marker concentration to obtain the titration curves which show a typical saturation equilibrium. These determinations carried out by exciting the sample at 390 nm, where the value of maximum fluorescence intensity (measured at a wavelength of 460-480 nm range) indicates the concentration of marker linked to the surface hydrophobic sites of proteins.

Fluorescence spectroscopy

Fluorescence measurements were carried out in pre- and post- adsorption phase either adsorbed to the nanoparticles or on oil droplet surface. Moreover the fluorescence of the different phases "cream" and “serum” phases were also analyzed.

Following are shown the different methods of preparation of the samples subjected to this analysis. They were conventionally divided into three types:

1. BLG fluorescence spectra were acquired from a sample protein concentration of 0.05 mg/mL suspended in 0.05 M sodium phosphate buffer, pH 7.
2. Complex BLG/NP: The fluorescence spectra were acquired from sample with protein concentration of 0.05 mg/mL.
3. BLG in the emulsion: to analyze these samples front-face technique was exploited.

The front-face fluorescence technique can conduct investigations on the structural proteins directly employing complex matrices, such as emulsions. The emulsions were prepared according to the criteria described in detail above. The analyzed samples were: whole emulsion, the aqueous phase "serum" and the lipid phase "cream". The cream and the

emulsion were diluted in a 1:2 ratio with 0.05 M sodium phosphate, pH 7, whereas the serum in a 2:1 ratio with the same buffer.

The measurements were performed in Perkin-Elmer LS 50B spectrofluorimeter equipped with a cell holder for measuring the fluorescence of solid matrices. Spectral parameters were: λ_{ex}: 280 nm, λ_{em}: 300-500 nm $Slit_{ex-em}$: 2.5 nm, Scan speed: 50 nm/min.

Trypsin limited proteolysis

Limited proteolysis experiments were performed as follows: 0.01 ml of trypsin solution (TCPK treated, 1mg/ml in 0.025 sodium acetate, pH 4.5) were added to 1 ml of BLG solutions, “cream” and “serum” samples (in 0.05 M sodium phosphate, pH 7, final protein concentration: 1mg/ml) in order to reach a ratio protease/substrate of 1/100 w/w. Hydrolysis were carried out for 30 min at 37° C and stopped by addition of 0.02 ml of Soybean Kunitz Tripsin Inibitor (2 mg/ml in dd water).

For hydrolysis of nanoparticles bounded BLG (final protein concentration: 0.05 mg/ml) the same protocol was followed, adjusting the amount of protease in order to respect the protease/substrate ratio (1/100 w/w by addiction of 0.005 ml of a 0.1 mg/ml TCPK treated trypsin solution).

In order to extract the resulting peptides after the hydrolysis one volume of each sample was treated with 3 volumes of hexane and 2 volumes of acetone in a glass vial. After vigorous mixing and subsequently separation of the two immiscible phases, the upper phase (the apolar one, containing the hexane and the extracted lipids or polystyrene) was removed. This step was repeated three times. The acetone from the delipidized polar phase was removed by vacuum stripping, and then the samples were lyophilized.

Peptides characterization by RP-HPLC

In order to characterize the peptides released from trypsin action, RP-HPLC was also employed. To this purpose, lyophilize samples were dissolved in an appropriate volume of buffer A (dd water, 0.1% TFA) and after centrifugation (15 min, 13.200 *x g*) loaded into the HPLC system (Waters 515, equipped with Waters 717 Autosampler and Waters 996 PDA detector). All the HPLC runs were performed using a Waters Symmetry 300Å C18 column. Running buffers were A (dd water, 0.1% TFA) and B (100% acetonitrile, 0.1% TFA). Flux was set at 0.8 ml/min with a separation gradient 0 to 100% buffer B in 125 min. Signals were recorded at 220 and 280 nm.

Peptides characterization by MALDI-TOF mass spectroscopy

The lyophilized samples were dissolved in dd water with 0.1 % TFA. After a cleaning passage, performed with a C18 Zip Tip devices (Millipore) using the protocol provided by the manufacturer, samples were mixed with either matrixes (α-cyano- 4-hydroxycinnamic acid or sinapinic acid) and loaded on the MALDI plates. MALDI-TOF mass spectrometry was carried out on a PerSeptive BioSystems Voyager DE-Pro spectrometer equipped with a N_2 laser (λ 337 nm, 3-ns pulse width, 20-Hz repetition rate).

The instrument operated with an accelerating voltage of 20 kV. External mass calibration was performed with low-mass peptide standards including angiotensin I, (m/z ¼ 1296.68), bovine α^{s1}-casein 1-23 peptide (m/z ¼ 2764.55) and bovine insulin (m/z ¼ 5730.61). Mass spectra were acquired in the reflector mode using Delay Extraction (DE) technology, and analyzed by using the software provided by the manufacturer.

Results

Determination of BLG stabilized emulsion droplets size

The procedure of emulsion formation (5% oil, 0.5% BLG, 5 cycles of sonication of 1 minute each) used from us allowed to produce emulsions with a relatively sharp size distribution, with an average droplet size of 0.249 μm. In Figure 3.6, is reported the graph which describes the particle size distribution of our sample.

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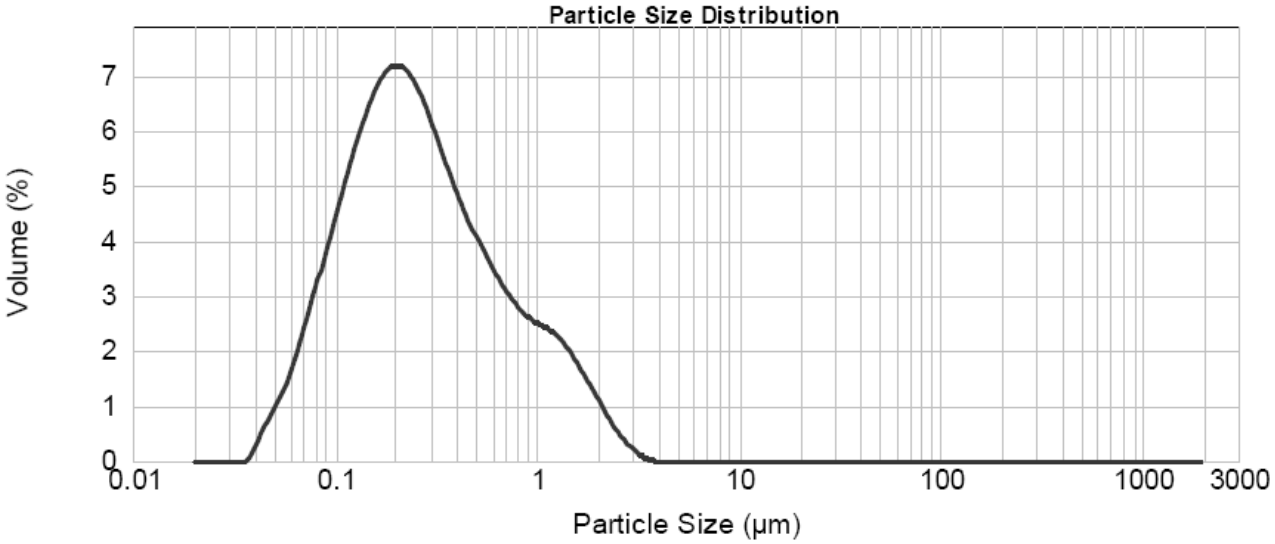

Fig 3.6: *particle size distribution of soy oil emulsions stabilized by BLG. Oil concentration 5% w/w, BLG concentration 0.5% w/w*

Determination of BLG adsorption ratio on emulsion nanodroplets

It is fundamental to estimate the protein amount adsorbed on the oil droplets every time a new protocol of emulsification was developed. This because the droplets size influences the amount of surfactant (in our case BLG) adsorbed on the droplet surface; the smaller emulsion droplets, the higher is the overall oil surface and consequently, the higher the request of protein to stabilize the biphasic system.

There are different ways to perform these measurements, that all requiring the prior separation of the two phases. Then the estimation takes place on the aqueous phase, using the common techniques of protein concentration determination, such as UV absorption at 280 nm (if only one protein is present as stabilizer) or colorimetric assays (BCA, Bradford or Lowry). The protein amount adsorbed on the oil droplets is then estimated by difference with the protein present in the “serum” phase. These methods are all affected by the facts that in the aqueous phase are always present substances that can

interfere with the measure, and there is not a direct information on the protein content in the "fat" phase.

To by-pass all these problems an SDS-PAGE quantification could be performed. This technique, if well performed, gives a reasonably accurate result, with a direct quantification of the protein cont in both fat and aqueous phases.

In Figure 3.7 is reported the SDS-PAGE gel of the protein quantification on the oil droplets of the emulsion made according the protocol reported above.

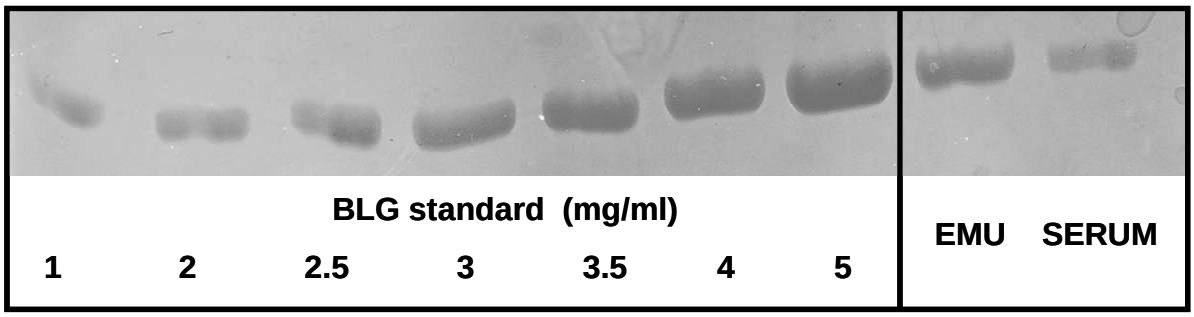

Fig 3.7: *SDS-PAGE of fat (EMU) and aqueous (SERUM) phases. In the left panel is shown the calibration curve*

After image digitalization and image analysis performed using a specific program (Image Master 1D, GE Healthcare), results that, in our protocol of emulsification approximately the 65% of the overall protein is present on the oil droplet surface, whereas 35% of the protein is still present free in the aqueous phase.

Study of adsorption isotherms of BLG on polystyrene nanoparticles

The adsorption isotherms were calculated using increasing concentrations of BLG with constant volume of nanoparticles (25 μl/ml) in 0.05 M sodium phosphate, pH 7. Figure 3.8 shows the adsorption isotherms obtained at 25 °C.

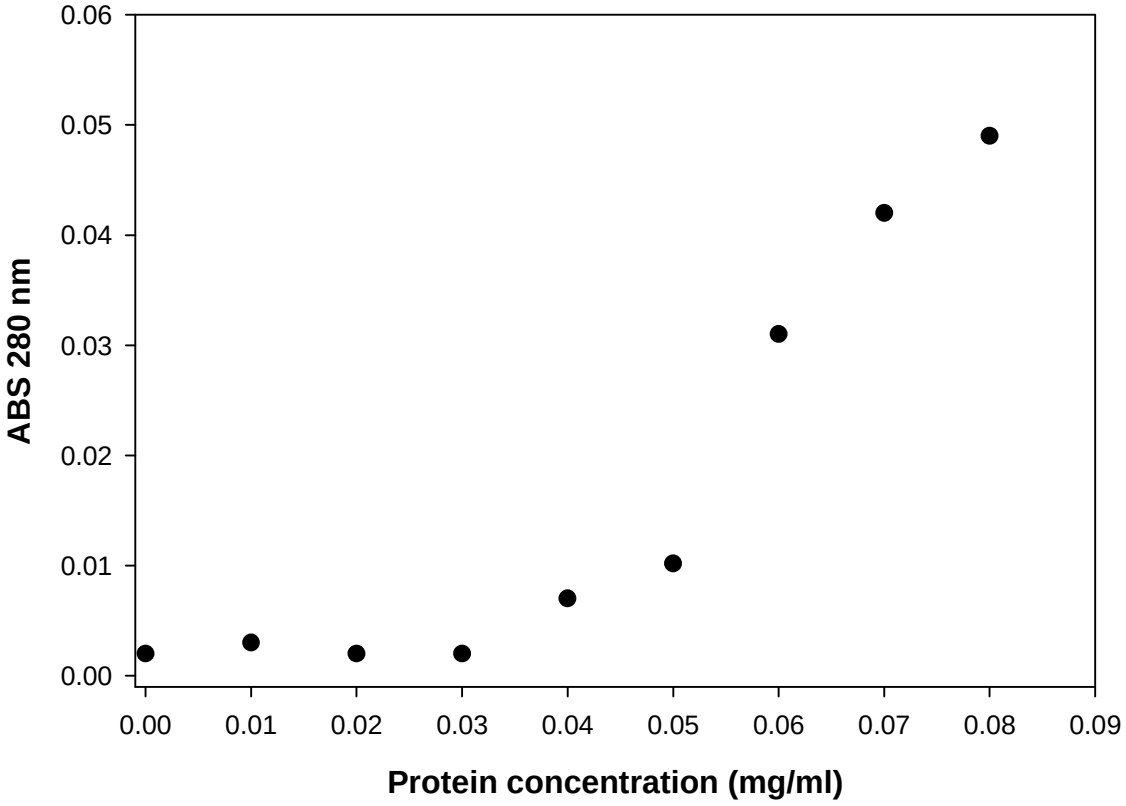

Fig 3.8*: adsorption isotherms of BLG addition of polystyrene nanoparticles in sodium phosphate buffer 0.05 M, pH 7.0 after incubation for 1 hour.*

A concentration of 0.05 mg/mL of β-lactoglobulin was chosen on the basis of adsorption tests performed. Under these conditions all the protein appears to be adsorbed to the hydrophobic surface of the NP and virtually no BLG is present free in solution.

The protein-NP association is essentially stabilized by hydrophobic interactions, as there are not to underestimate electrostatic forces that may play an important role in the orientation of protein molecules.

Structural organization of BLG at the NP surface

Intrinsic fluorescence measurements were used to analyze the structural organization of the protein adsorbed at the interface and to highlight any changes in the protein hydrophobic core. In particular, it was decided to perform fluorescence measurements on the native protein and the protein adsorbed to nanoparticles. In both cases the spectra were recorded at temperatures of 20° C, 30° C, 40° C, 50° C, 70° C, 80° C, and at 20° C after cooling the sample preventely heated at 80° C. These spectra were recorded

between 300 nm and 500 nm, exciting the sample at 280 nm, taking advantage of the characteristic fluorescence of tryptophan residues. There are in fact, in BLG, two tryptophan residues, which are used as fluorescence markers of structural organization: Trp_{19} and Trp_{61}. The second is located in the outermost position, near the disulfide bridge Cys_{66}-Cys_{160}, which acts as a strong quencher. Trp_{19} is located at the base of the hydrophobic calyx inside the barrel, and inaccessible to the solvent. This tryptophan is conserved in the lipocalin family and may have a structural role in BLG folding. This compound is believed to be responsible for more than 70% of the fluorescence signal (Fessas *et al*., 2001).

Figure 3.9 shows the fluorescence spectra of the native protein and of BLG bound to latex nanoparticles. The spectrum of the BLG in solution shows a maximum emission at 340 nm, typical of the native protein. This shows that Trp_{19} is located within the structure of hydrophobic protein. It also shows how the structure changes when the protein BLG is located at the interface of the hydrophobic NP. The shift of the peak emission to longer wavelengths indicates a greater exposure of the hydrophobic calyx of the protein, where the Trp_{19} residue is located. This would result in a change in the tertiary structure of adsorbed protein that allows increased exposure of Trp_{19} to the aqueous solvent.

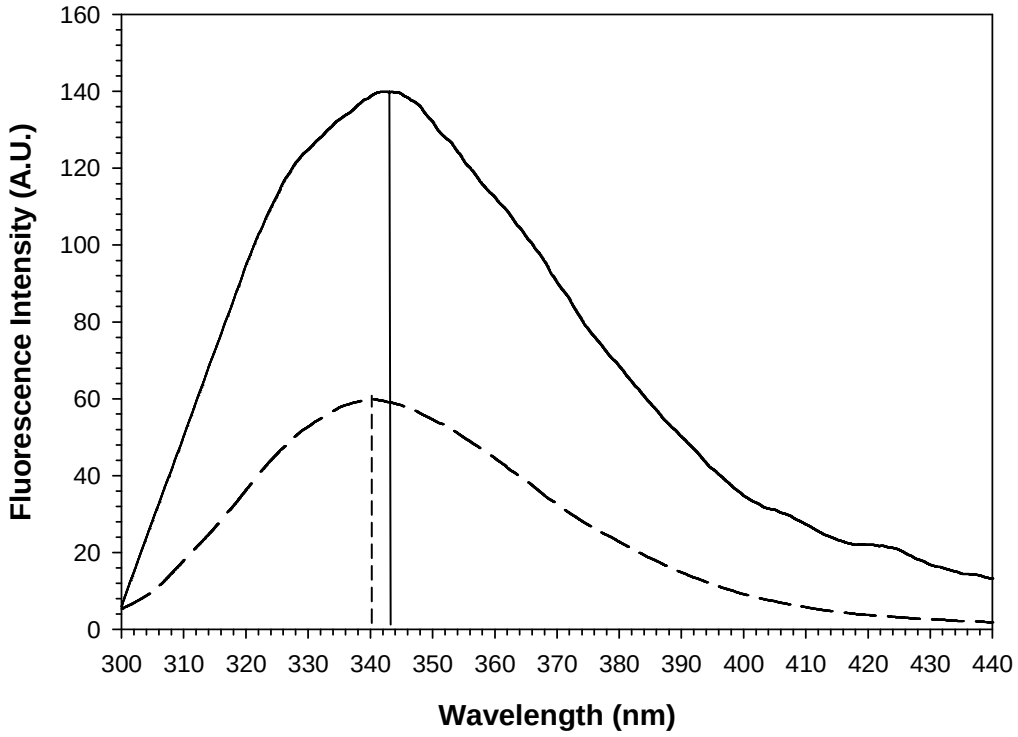

Fig 3.9: *tryptophan fluorescence spectra (λ_{ex}: 280 nm) of BLG free in solution (dashed line) and adsorbed on the NP surface (solid line)*

We then decided to study the protein intrinsic fluorescence, both free in solution and stacked to the NP interface with step-wise process of heating and subsequent cooling to 20° C. It is clear that BLG in solution changes its structure after a heat treatment at 80° C (Figure 3.10). The protein shows a shift in the maximum emission peak due to increased exposure of the hydrophobic core to the aqueous solvent. The protein is denatured and it loss the tertiary structure typical of native BLG in irreversible fashion.

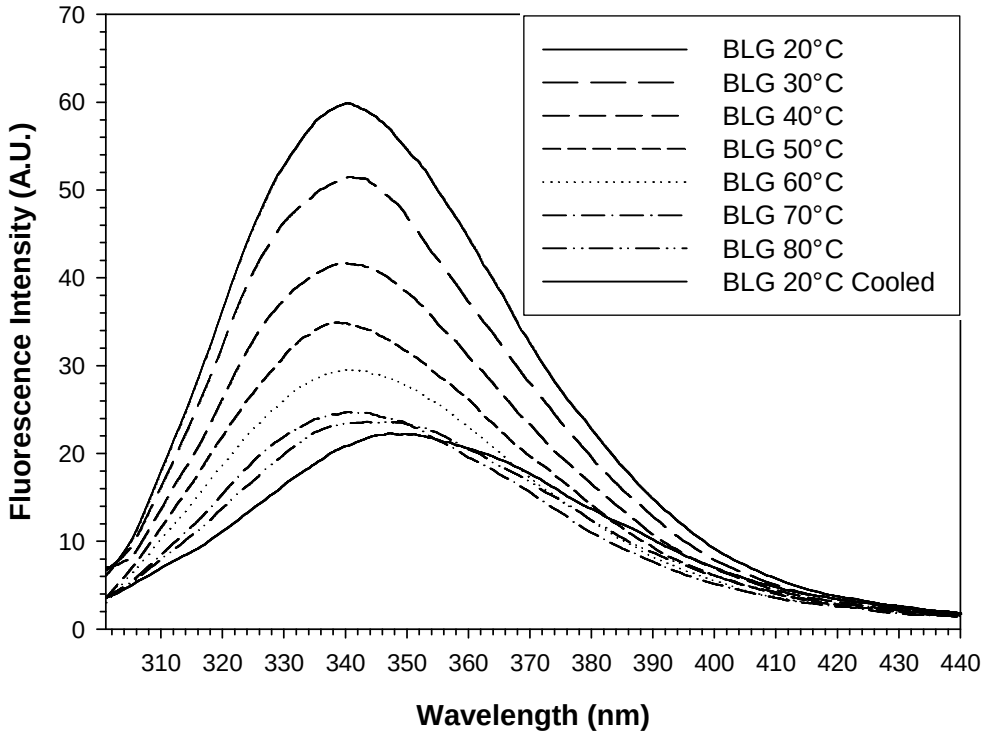

Fig 3.10: *tryptophan fluorescence spectra (λ_{ex}: 280 nm) of thermal denaturation ramp of BLG in solution*

Conversely BLG retains its modified structure even after heat treatment at 80° C when bound to NPs surface. During the heating process the NP-bound protein does not undergo structural changes and the interaction with NP can help to maintain the newly acquired tertiary structure once adsorbed (Figure 3.11).

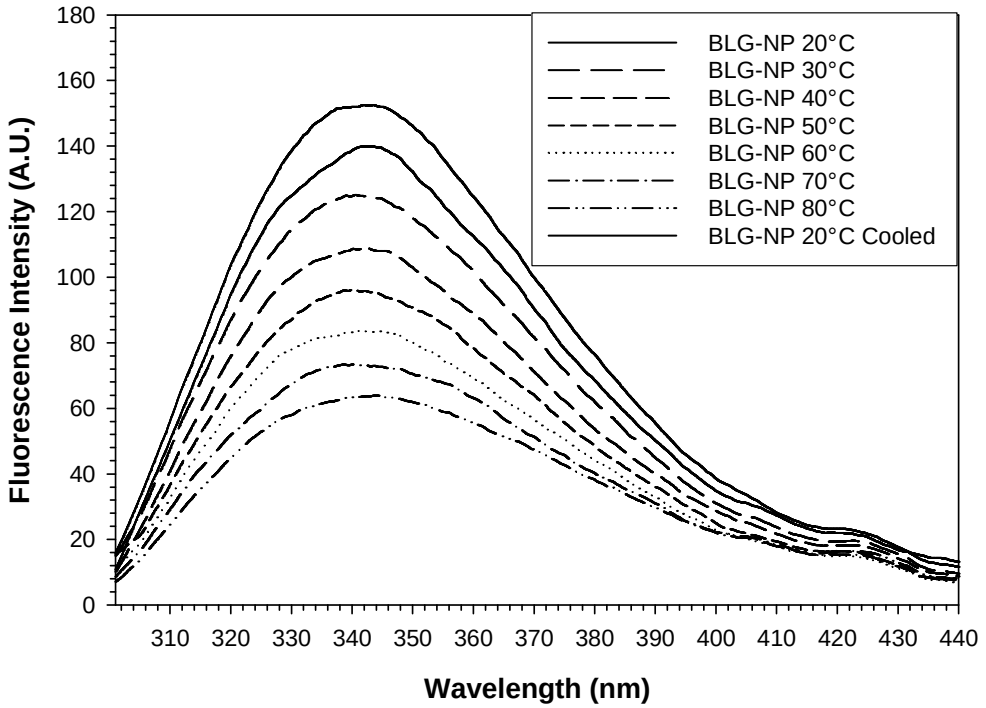

Fig 3.11: *tryptophan fluorescence spectra (λ_{ex}: 280 nm) of a thermal denaturation ramp of NP-bound BLG*

It is clear the behavior differences in thermal stability when the protein is in solution and when adsorbed to nanoparticles. The protein in solution shows a clear shift of the emission peak maximum after heating whereas it is not present in the BLG when adsorbed to the nanoparticles interface.

In summary, despite the importance of hydrophobic interactions of adsorbed BLG, we can not affirm that the tryptophan residues are located in close contact with the hydrophobic surface of polystyrene. This thesis is supported by the evidence of a "red shift" of the fluorescence emission maximum, indicating that one or more tryptophan residues are exposed to the solvent. A number of hydrophobic residues must be in close contact with the hydrophobic polystyrene interface to can be realized hydrophobic interactions. The new spatial arrangement of these hydrophobic residues in contact with the surface of the nanoparticles results in a specific conformation of the BLG interface and about the new arrangement of tryptophan residues.

By studying the structure of the protein we can get information about the preferential orientation of the hydrophobic portions of the interface. At pH 7 both BLG and nanoparticles have negative charge (even though this king of nonsupport has a “plain

surface", a few numbers of sulphate negatively charged groups still remain from the polymerization step), so portions of the protein that contain negatively charged will be exposed to the solvent and will turn away from the surface of polystyrene. The region of the protein involved in binding with the hydrophobic nanoparticles could be the highly hydrophobic part between the α-helix and β-barrel (Figure 3.12).

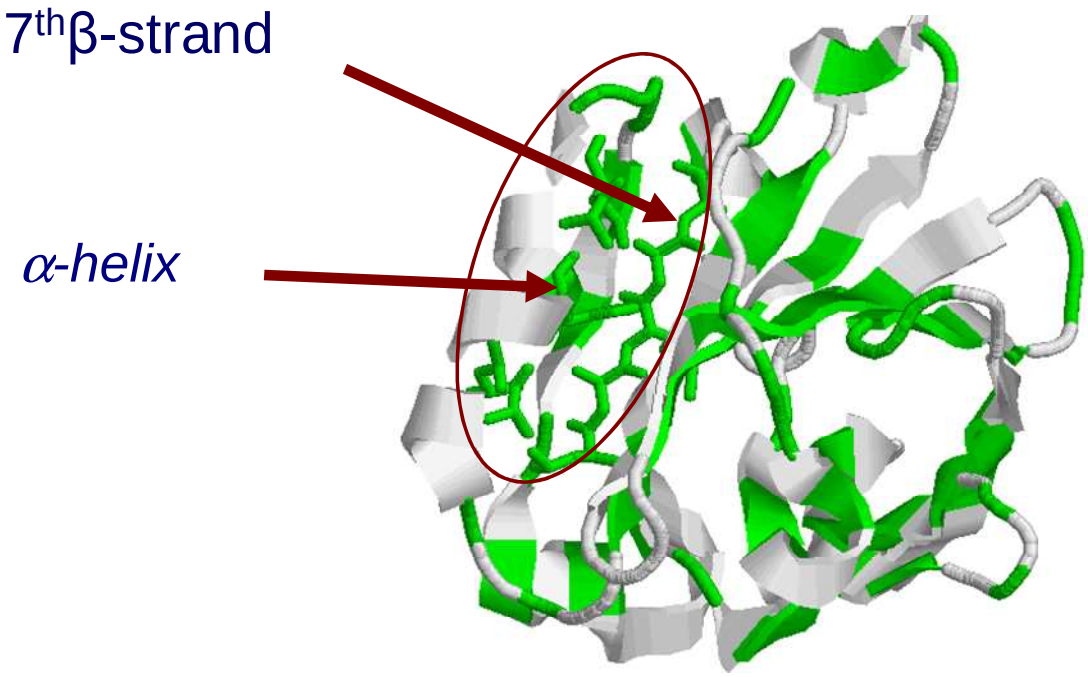

Fig 3.12: *hypothesized BLG interaction surface with a hydrophobic surface (pdb entry: 1UZ2)*

In addition, the adsorbed protein shows a 2.5 fold increase in fluorescence intensity compared to the free protein. The explanation of this phenomenon of protein global quantum yield gain can be attributed to two different hypotheses: either by the moving of the Trp_{61} from the disulfide bridge Cys_{66}-Cys_{160} (Figure 3.13), or by removal of Trp_{19} from Arg_{124} which is located above the Trp_{19} indole ring, which can act as quencher (Figure 3.14). Both these cases imply a stretching of the original structure.

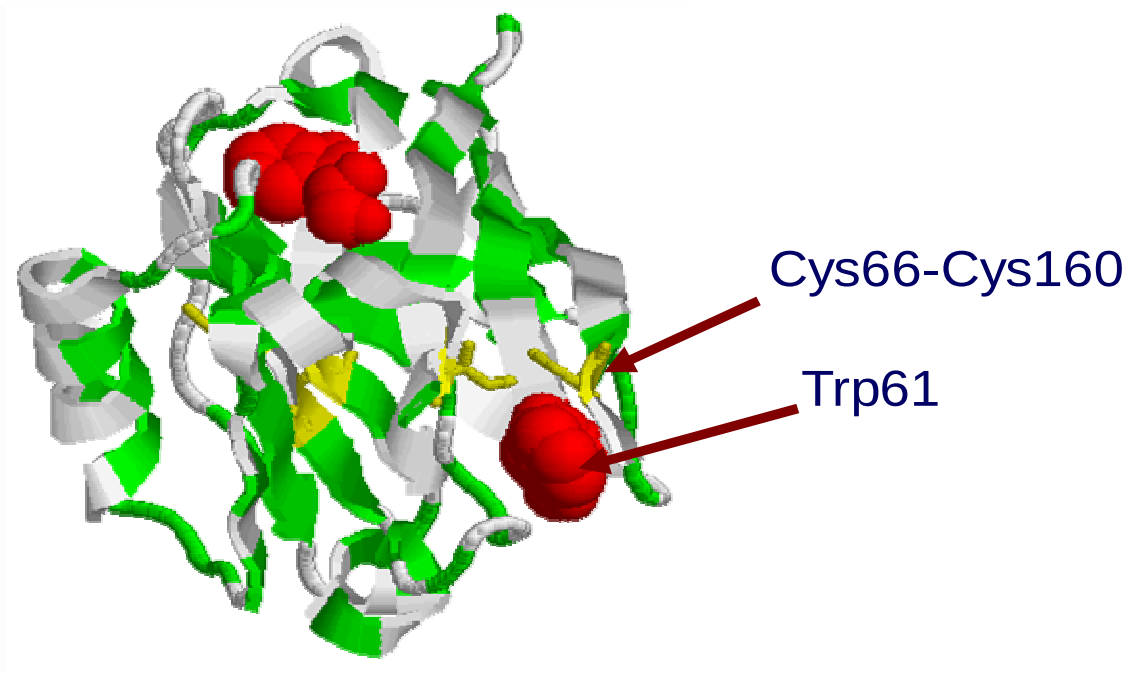

Fig 3.13: *3D structure of BLG (pdb entry: 1UZ2) with highlighted Trp_{61} and the disulfide bridge Cys_{66}-Cys_{160}. The removal of the fluorescent residue from the disulfide bridge may explain the increase of BLG fluorescence intensity after adsorption on NP*

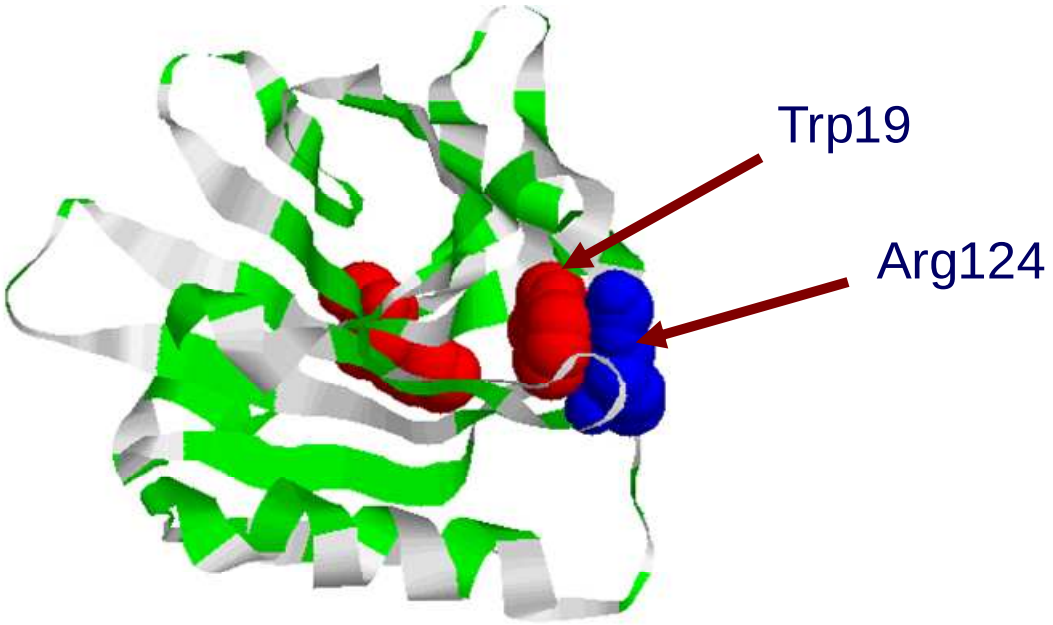

Fig 3.14: *3D structure of BLG (pdb entry: 1UZ2) with highlighted Trp_{19} and Arg_{124}. The removal of the fluorescent residue from the natural quencher may explain the increase of BLG fluorescence intensity after adsorption on NP*

Study of β-lactoglobulin structural reorganization in emulsion by front face fluorescence

In addition to studying the BLG behavior at NP interface we wanted to try and also to describe the structural features of the protein present at the surface of oil in water nanoemulsion. For these purposes we used front face fluorescence as the investigation method technique.

This technique allows direct fluorescence measurements on solid or multiphase matrices (Genot *et al*., 1992), and therefore also on liquid-liquid emulsions. In particular, intrinsic fluorescence measurements were performed to analyze the structural organization and structuring properties of BLG in nanoemulsions made with vegetable oil (soybean oil) compared with BLG in aqueous solution. Specifically studies were carried out on the different phases obtained by centrifugal separation and on the emulsion itself. Spectra were recorded between 300 and 500 nm, exciting the sample at 280 nm, thus taking advantage of the characteristic fluorescence of tryptophan residues. Figure 3.15 shows the fluorescence of tryptophan in the case where the BLG is in the emulsion phase and cream phase serum at room temperature, showing a different structure of the protein when adsorbed or present in solution.

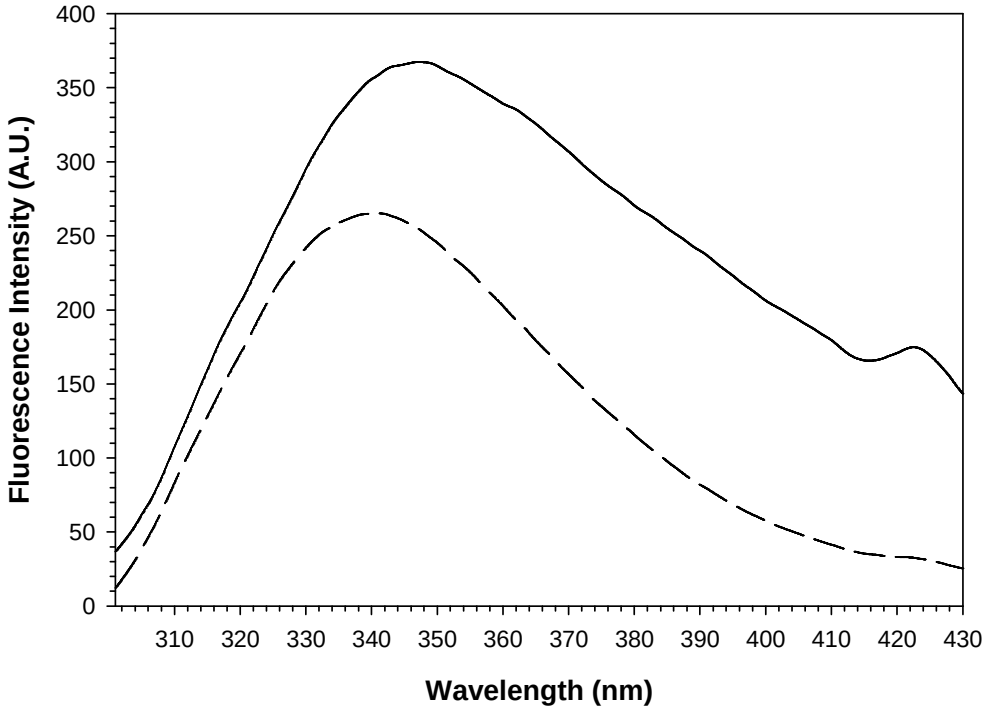

Fig 3.15: *tryptophan fluorescence spectra (λ_{ex}: 280 nm) of BLG free in solution (dashed line) and adsorbed on the oil droplet surface (solid line)*

Whereas the protein in the serum phase shows a peak emission comparable to that of the native protein, it is clear a shift of the peak of the tryptophan emission maximum towards longer wavelengths such as in the case of the emulsion and the cream (Figure 3.16).

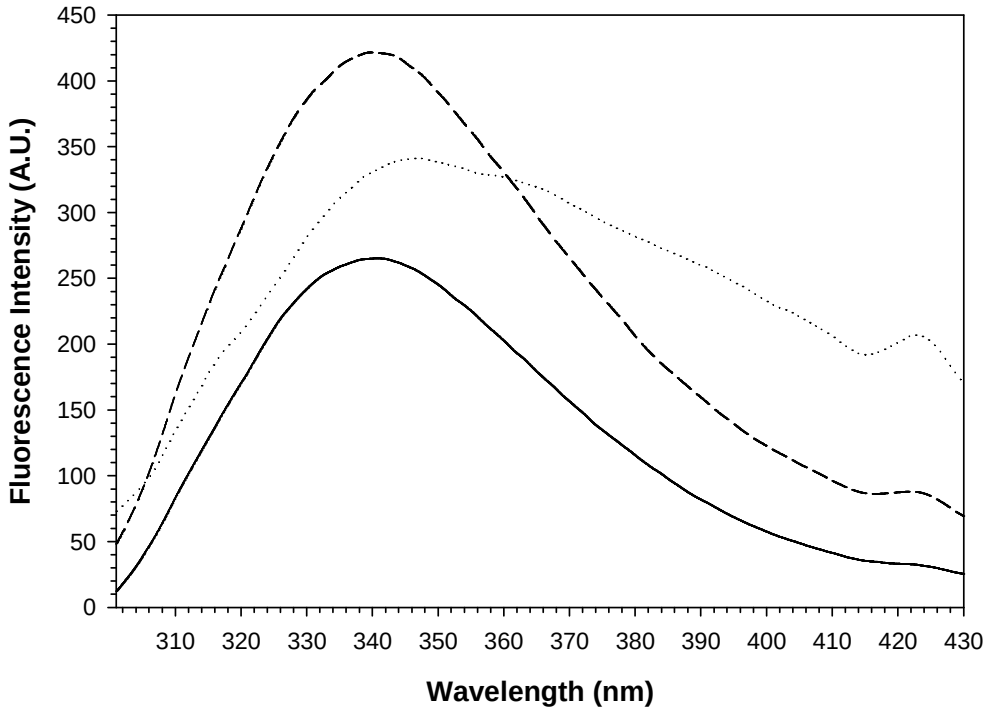

Fig 3.16: *tryptophan fluorescence spectra (λ_{ex}: 280 nm) of BLG free in solution (solid line), BLG on oil droplets (dotted line) and BLG in the emulsion aqueous phase (dashed line)*

This is a typical behavior of tryptophan, whose exposure to aqueous solvent results in the so-called "red shift" of the emission maximum compared to the situation in which the tryptophan is "buried" within the structure of the protein. Unfortunately no reliable information can be obtained from these spectra intensity analysis.

This shows that the protein on this kind of hydrophobic surface, as is the case in the study of interaction with nanoparticles, takes a different spatial conformation favoring hydrophobic type interactions.

Study about BLG hydrophobicity properties after adsorption on nanoparticles

Many studies have pointed out that the only tryptophan fluorescence is not sufficient to provide all the information about structural changes affecting the protein hydrophobic regions, when they change their properties of surface hydrophobicity (Eynard *et al*. 1992;

Iametti *et al*., 1998). Changes of surface hydrophobicity properties of native and surface unfolded β-lactoglobulin can be evaluated by spectrofluorimetry, using appropriate hydrophobic markers, such as 1-anilino-8-naphthalene sulfonate (ANS), capable to become fluorescent after binding to a hydrophobic site on the proteins surface (Genot *et al*., 1992, Pagani *et al*., 1998).

Tests were conducted initially in solutions with constant protein concentration (0.05 mg/mL) containing increasing amounts of ANS. These determinations were carried out by exciting the sample at 390 nm, allowing constructing the titration curves shown in Figure 3.17, where the value of maximum fluorescence intensity indicates the amount of marker bound to the surface hydrophobic sites of proteins.

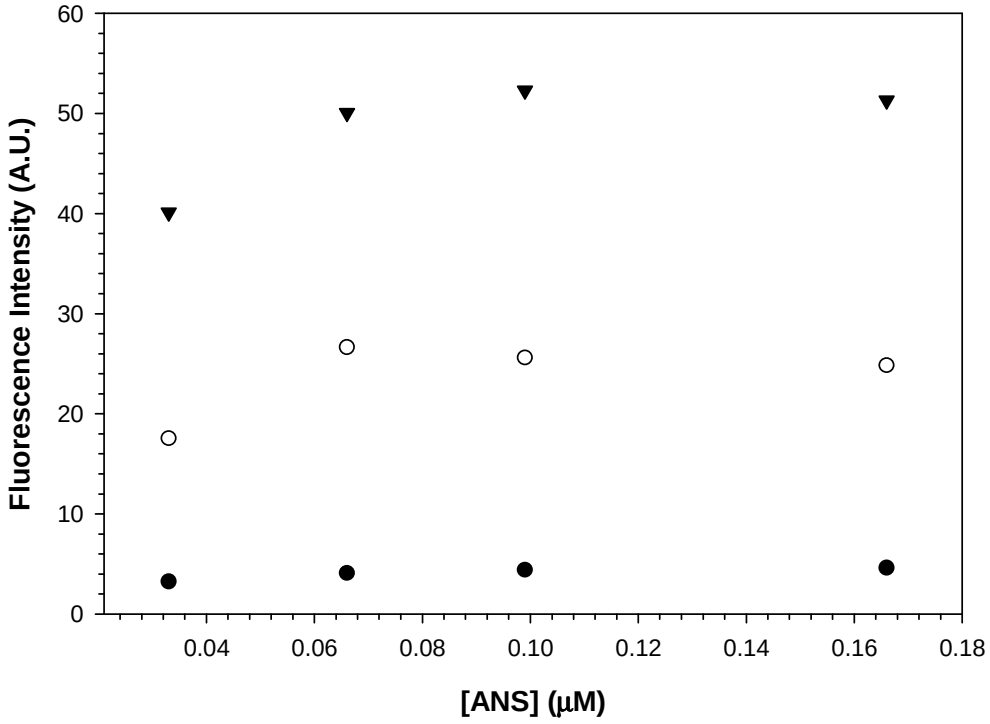

Fig 3.17: *ANS fluorescence intensity (λ_{ex}: 390; λ_{em}: 460-480 nm) of BLG free in solution (black circle), latex nanoparticles alone (white circles) and BLG adsorbed on NP surfaces (white triangles)*

The titration curves confirm the significant differences between the native protein and that adsorbed to the NP, as well as with regard to the hydrophobic surface of the nanoparticles in the same state (Figure 3.17). It is very clear that the surface hydrophobicity of BLG increases when they are adsorbed to the polystyrene nanoparticles. The structural reorganization of the protein present at the interface allows a greater exposure of the hydrophobic core, already highlighted by studying the fluorescence

properties of tryptophan in the same sample. We can therefore conclude that the presence of hydrophobic solid-liquid interface results in significant changes in the tertiary structure of the protein and facilitates the saturation of surface sites by the hydrophobic probe.

Accessibility changes of Cys_{121} after protein adsorption on nanoparticles

Variations in the structural organization of the BLG were further investigated by measuring the accessibility of thiol residues. Each monomer contains five cysteine residues which four are engaged in two disulfide bridges (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}). These covalent bonds stabilize the structure. The only free thiol (Cys_{121}) in the native protein structure is hidden between the β-barrel and the α-helix.

This parameter is very useful to show any changes in the protein structure and to investigate possible post-adsorption reorganizations. The study was conducted using a specific reagent (DTNB, 5,5'-dithiobis-2-nitrobenzoic acid) at various temperatures: 25°C, 45°C, 60°C and 70°C. The absorbance values at 41 2 nm, measured at different times by the addition of the reagent DTNB, allowed to determine the number of -SH groups readily available and the maximum number of accessible thiol residues when the equilibrium is reached. In experiments involving BLG in solution there is an increase of the absorbance values in relation to the temperature. At 45°C an increased free thiol exposure is observed because of accelerate unfolding. At 70° C the prote in expose almost completely and almost immediately the free thiol of Cys_{121}. We measured the accessibility of -SH groups in the BLG adsorbed to latex nanoparticles, and these results make it clear that the protein exposes its Cys_{121} thiol also at room temperature after the interaction with the nanoparticles. It is possible that the protein undergoes a change in the structure which improves the exposure of Cys_{121} thiol to the reagent. Table 3.1 reassumes all the exp carried out with this technique.

Sample	***[SH] (μM) titrable***	***% of Cys_{121} accessible***
β-LG 25℃	*0.0*	*0*
β-LG 45℃	*0.9*	*30*
β-LG 70℃	*3.1*	*100*
β-LG-NP_{46} 25℃	*3.1*	*100*

Tab 3.1: *accessibility of the thiol of β-LG in solution and bonded to nanoparticles, measured spectrophotometrically at 412 nm with DTNB (molar absorbance equal to 13600 M^{-1} cm^{-1}).*

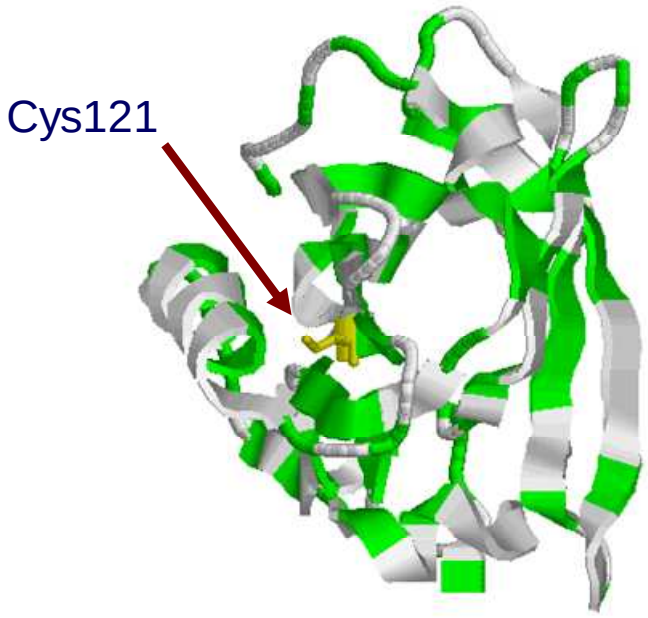

Fig 3.18: *3D structure of BLG (pdb entry: 1UZ2) with in evidence the Cys121, residue that become accessible after interaction with the nanoparticles surface*

Taken together, all these results allow us to hypothesize a possible BLG conformation on the latex nanopartilcles, in which mostly of the protein present interact with the hydrophobic surface with the area directly at the opposite of the alpha-helix and the ninth beta-strand. In this position, Cys_{121} become accessible, both Trp moves away from their natural quenches and the overall hydrophobic accessible surface become accessible.

RP-HPLC characterization of hydrolyzed BLG

In order to have more exhaustive information about the regions interacting with the hydrophobic interface, we have performed limited proteolysis using trypsin as protease. We performed these hydrolysis on BLG free in solution and on adsorbed BLG either at the emulsion interface or on latex nanoparticles.

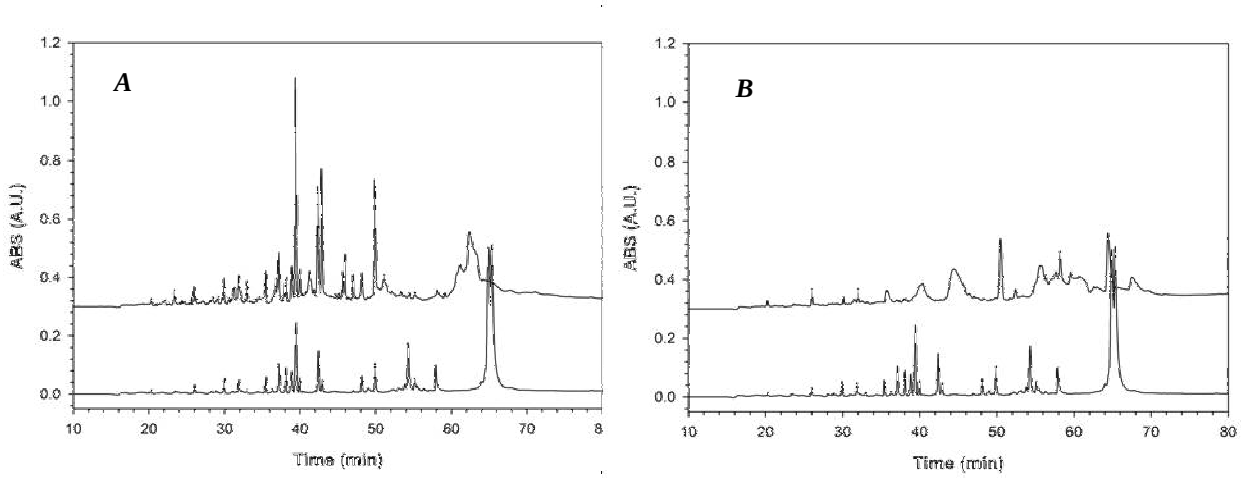

Fig 3.19: *RP-HPCL tracers for (**panel A**) peptides from tryptic hydrolysis of free BLG (bottom chart) and emulsion BLG (top chart) and (**panel B**) peptides from tryptic hydrolysis of free BLG (bottom chart) and nanoparticles/BLG (top chart)*

As shown in Figure 3.19A it is clear that the adsorption process makes the protein more susceptible to proteolysis. The peak corresponding to the native BLG (peak at ≈ 65 min) decreases in the emulsion sample. Moreover, after adsorption, BLG shows new cutting sites, unaccessible when the protein is in its native form. This results in a formation of new peptides, observable in the upper top chart in the Figure 3.19A. This BLG behavior to protease was previously described by Malaki *et al*, 2010.

We also performed hydrolysis on BLG adsorbed on latex NP. Also in this case (Figure 3.19B) the hydrolysis gave different peptide pattern, compared with the free BLG. The hydrolysis in this physical form results in almost complete disappears of native protein.

Comparing the peptides from the two different interfaces, we can see how the liquid/liquid and solid/solid surface act in different way on the protein structure.

Mass spectroscopy characterization of BLG hydrolyzed

In this study we also characterized the peptide analyzed in the previous paragraph by MALDI-TOF mass spectroscopy. Spectra were recorded either in α-cyano- 4-hydroxycinnamic acid or sinapinic acid in order to test all the mass range (from small peptides to the whole protein). Generally, spectra recorded in sinapinic acid were blank, meaning an absence of high mass peptides. This is true for exception of peptide from BLG/nanoparticles. A good number of peptide, especially from nanoparticle sample does

not come from “classic” tryptic cleavage. In the Tables below are reported the peptides identified in the various experimental conditions, and are reported in the note the possible variants and adducts.

Measured molecular mass (Da)			***Corresponding sequence***	***Note***
Free BLG	***Emulsion***	***Nanoparticles***		
487.56			***9-13***	
674.42	**674.4**		***78-83***	
701.45	**701.4**		***70-75***	
	802.5		***71-77***	
837.47	**837.47**		***142-148***	
903.56	**903.56**		***76-83***	
916.47	**916.5**		***84-91***	
933.54		**935.18/978.7**	***1-8***	K^+
1065.58			***98-100***	
		1153.6	***139-148***	
1192.54			***61-70***	
1245.58	**1245.6**		***125-135***	
1635.77	**1635.77**		***125-138***	
1644.83			***149-162***	
1658.78	**1658.78**	**1661.73**	***149-162***	
	1916.32		***1-18***	
2030.34		**2032.93**	***21-40***	
2313.25		**2310.38**	***41-60***	
2647.2			***102-124***	
	2651.07		***102-124***	***Var A***
2707.37			***15-40***	
	2722.15	**2769.95**	***61-69-S-S-149-162***	***Disulphide***
3486.78			***41-70***	
	3359.82		***41-69***	
	3362.87		***9-40***	
		6573.87	***61-70-S-S-139-162-S-S-139-162***	***2 Disulphide***
		8226.55	***102-162-S-S-61-70***	
		8572.67	***102-162-S-S-61-70***	***+ lactose***

Tab 3.2: *list of masses and sequences of the tryptic peptides generated from BLG present in solution, at emulsion or at nanoparticles interface*

Conclusions

The study we performed was aimed at creating a useful model to understand some of the structural rearrangements of the β-lactoglobulin when adsorbed to an interface. Specifically, we wanted to investigate the behavior of BLG present at the solid-liquid (aqueous solution of nanoparticles) and liquid-liquid (vegetable oil in water nanoemulsion).

The binding of BLG occurred to nanoparticles has been studied by using spectrofluorimetric techniques. Intrinsic fluorescence measurements were used to analyze the structural organization of the protein adsorbed at the interface and highlight any changes in the hydrophobic core. In particular, we studied the behavior of Trp_{19}, which lies at the base of the calyx hydrophobic inside the structure and unaccessible to solvent. The investigations carried out at room temperature have shown that the protein structure changes when the BLG is located at the interface of the hydrophobic latex NP. The shift of the peak emission to longer wavelengths indicates in fact a higher exposure to the solvent of the hydrophobic region of the protein, where the Trp_{19} residue is located. This would result in a change in the tertiary structure of adsorbed protein that allows increased exposure of Trp_{19} to the aqueous solvent. By analyzing the behavior of the protein at the nanoparticle interface we found that adsorbed BLG retains its modified structure even after heat treatment at 80° C. During the heating process the protein does not undergo structural changes as the interaction with the NP can therefore maintain the newly acquired tertiary structure once adsorbed. In addition, the adsorbed protein shows an increase of fluorescence intensity (2.5 times that of the free protein). The explanation of this phenomenon can be explained on the basis of to two different hypotheses: either the removal of the Trp_{61} from Cys_{66}-Cys_{160} disulfide bridge, or by removal of Trp_{19} from Arg_{124} which is located above the Trp_{19} indole ring, which can act as fluorescence "quencher" Either hypothesis implies a stretching of the original structure.

The study was extended to carry out also by analyzing the surface hydrophobicity of BLG-NP that increases when BLG interacts with polystyrene nanoparticles. The structural reorganization of the protein present at the interface allows a greater exposure of hydrophobic regions, already highlighted by studying the fluorescence properties of tryptophan in the same sample. One can therefore conclude that the presence of hydrophobic solid-liquid interface results in significant changes in the tertiary structure of the protein and facilitates the saturation of protein surface sites by the hydrophobic probe.

Accessibility of -SH groups of the BLG also increases when the protein is adsorbed on NP. Taken together, the determination of accessible -SH residues shows that the adsorption on latex nanoparticles is able to alter the behavior of the protein.

By studying the behavior of BLG present at the interface in emulsion, using front-face fluorescence techniques, we show a different structural organization of the adsorbed protein. While the protein present in the unadsorbed phase (serum) shows an emission peak comparable to that of the native protein, emulsion and the cream sample display a shift of the tryptophan emission maximum peak towards longer wavelengths. This shows that the protein at hydrophobic interface, as is the case in the study of interaction with nanoparticles, takes a different spatial conformation favoring interactions of hydrophobic type.

The results obtained from tests on protein hydrolysis in emulsion shows that adsorption of BLG makes it more susceptible to proteolysis. Once adsorbed at the interface liquid-liquid protein changes its structural conformation and is hydrolyzed much faster than the protein in solution. This suggests that the interaction induces conformational changes, allowing the protease to act much more selectively than is seen in the protein in solution. Moreover, also BLG hydrolysis on nanoparticles lead to different peptide pattern and a more efficient digestion of the protein. Unexpectedly, adsorbtions also appears to alter the amminoacidic specificity of the protease.

These results show the relevance of protein digestion behavior at interface because pointed out the different kinetics and modality of digestion, with the production of “unusual” peptides. Whereas these event may have physiological, nutritional, or immunological relevance remains to be assessed, as the possibility that the altered behavior of the adsorbed proteins (and the altered activity of the protease that acts on it) may have a broad significance in the case of food proteins.

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Chapter 4)

β-LACTOGLOBULIN STRUCTURAL REARRANGEMENT AT INTERFACES: RELEVANCE TO ITS IMMUNOGENIC BEHAVIOR

Introduction

Cow's milk contains about 30-35 g/l of proteins that are divided into serum proteins (approx. 20%) and casein micelles (ca. 80%). The whey contains protein derived from synthesis in the mammary gland such as α-lactalbumin (ALA) and β-lactoglobulin (BLG) and proteins such as blood serum albumin (BSA), lactoferrin (LF) and immunoglobulins. The casein component (CN) includes four fractions encoded by different genes on the same chromosome: α_{s1}-, α_{s2}-, β- and κ-casein.

It is important to note that allergy to cow's milk is not due to a single component or to a single protein fraction, but is characterized by the multiplicity and diversity of the involved proteins. It often happens that a multisensibilization might be present to multiple proteins simultaneously and, in general, all the milk proteins are considered potential food allergens, including those in which it is present in trace amounts (Gjesing *et al*., 1986; Docena *et al*., 1996). However, the main milk allergens are casein, BLG and ALA that are most important proteins in quantity in the milk. In recent years, the immunoreactivity to caseins seems to have grown both in terms of frequency and intensity of the IgE response (Stoger and Wutrich, 1993; Wal, 2002) and some research has shown that caseins may cause milk main persistent allergy (Sicherer, 1999). On the other hand protein also present in smaller quantities are of importance from the point of view of allergy. Wal and other authors (1995) have shown that approximately 35-50% of patients with BSA sensibilization, are also sensitive to immunoglobulins and lactoferrin.

The milk proteins are very heterogeneous and have little structural or functional characteristics in common. This heterogenicity is increased by their genetic polymorphism, which results in multiple variants for each protein. The variants are characterized by the replacement of missing amino acids or peptide fragments of different sizes, although altered pathway of post-translational modifications such as phosphorylation and glycosylation. All these modifications and other changes generated by processes of food preparation may affect the ability to bind with IgE and on allergenicity (Malik *et al*., 1988, Bernard *et al*., 2000).

β-lactoglobulin (BLG) exists naturally as a dimer of 36 kDa and each subunit has 162 amino acids. Together with alpha-lactalbumin (ALA), BLG is considered a major allergen present in the milk and the lack of a homologous protein forms in human milk has suggested that BLG was among the main cause of allergenicity. The BLG monomer contains two disulfide bridges (Cys_{106}-Cys_{119} and Cys_{66}-Cys_{160}) and a free cysteine

(Cys_{121}). The latter, together with the bridge Cys_{106}-Cys_{119} is more internal located than the Cys_{66}-Cys_{160} bridge, that is exposed outside near the C-terminal region. This type of structure is responsible for the main physico-chemical properties and interactions that are established with casein during heat treatment. Structural and physico-chemicals properties of BLG were discussed in more detail in the previous chapter. The protein has a proved resistance to acid and enzymatic hydrolysis, and this allows it to be absorbed virtually intact from the intestinal mucosa. The biological function of BLG is to bind retinol (vitamin A) and transfer it from breast milk to the infant through specific receptors in the gut. In cow's milk there are eight genetic variants of BLG. Variant A and B are the most abundant and the most common (Vasbinder, 2002).

It is not possible to establish a general relationship between structure and allergenicity of milk proteins. Some of the IgE-binding studies have shown the presence of sequential epitopes vary in size and even peptides of 12-14 amino acid residues (about 1500 Da) that appear to play a significant part of the whole molecule allergenicity in some patients.

There is not still a clear picture about the consequences of technological treatments, and in particular of thermal, on allergenicity of cow's milk protein. The results depend on the temperatures and heating times and the possible interaction with the food matrix. It is important to note that although the thermal denaturation results in destabilization of the native structure that does not mean that there is loss of allergenic potential. During treatment it may come and form aggregates that increase the risk of allergenicity. Some authors have compared the raw milk with pasteurized or pasteurized and homogenized, and observed that during the heat treatment did not have reductions in allergenicity (Gjesing *et al*., 1986, Host and Samuelsson, 1988; Werfel *et al*., 1997).

On the other hand, it is generally accepted that the hydrolysis of milk proteins reduces considerably their allergenicity although several studies have shown that specific IgE to cow's milk allergic patients recognize, sometimes to a greater extent, products of enzymatic chopping. (Haddad *et al*. 1979; Spuergin *et al*., 1996, Maynard *et al*. 1997; Selo *et al*., 1999).

Aim of the work

The aim of this work is to understand whether and how the β-lactoglobulin conformational changes derived from the interaction with hydrophobic interfaces, can modulate its immunoreactivity and its absorption behavior by human cells involved in the immuno response.

Materials and methods

Chemicals

All reagents used were in the highest degree of purity commercially available, and purchased from Sigma Aldrich unless otherwise specified. Nanoparticles (average size: 46 nm) were provided by Kisker Bioteck.

β-lactoglobulin purification

To avoid the presence of the partially denatured β-lactoglobulin species that characterize the commercial preparations we proceeded to purify the protein directly from fresh milk whey. The purification was performed by ion exchange chromatography of the serum obtained by skimming and subsequent removal of the casein fraction.

Whole milk was skimmed by centrifugation at 5000 *x g* for 30 min at 4° C. Skimmed milk was then filtered on gauze to remove any remaining cream. The casein fraction was removed exploiting isoelectric precipitation by acidification to pH 4.5 with 5 M HCl. The precipitated casein was removed by centrifugation at 7000 *x g* for 5 min at 30° C. The serum obtained was then neutralized to pH 7.2 by addition of TRIZIMA base powder. To remove all the remaining portion of casein, whey was centrifuged at 11000 *x g* for 60 min at 20° C.

The separation of BLG from serum was obtained by ion exchange chromatography. An appropriate volume of serum is diluted with water in 1:1 ratio to reduce the ionic strength and then loaded onto a DEAE-cellulose (Whatman) packed column previously equilibred in 0.05 M Tris-HCl, pH 7.2.

BLG and many other serum proteins, including α-lactalbumin (ALA), are negatively charged and bind to the resin, while other proteins with different charge (lactoferrin, lactoperoxidase, lysozyme, etc.) are eluted in the unbound fraction. BLG was eluted from

the resin using a step gradient of NaCl. The first step consists of 0.05 M Tris-HCl, pH 7.2, containing 0.1 M NaCl in which the ALA is eluted, followed by a second step in which the BLG is eluted using a buffer containing 0.3 M NaCl. The purified BLG was then concentrate using an Amicon ultrafiltration devices (Millipore) with a membrane of 10 kDa cut-off, and subsequently lyophilized.

Protein concentration determination

The protein concentration of the different samples was determined spectrophotometrically by measuring the absorbance at 280 nm, using a 1 cm quartz cuvette in an Perkin Elmer Lambda 2S spectrophotometer. The concentration in mg/mL was calculated using an extinction coefficient, ε^{280} of 0.93 mg ml^{-1} cm^{-1}.

FITC β-lactoglobulin conjugation protocol

FITC is among the most simple and commonly used reagent for protein fluorescent labeling. This isothiocyanate fluorescent derivate reacts with amino, sulfhydryl, imidazoyl, tyrosyl, or carbonyl groups on proteins. However, only the derivatives of primary and secondary amines generally yield stable products. Reactions are most efficient at pH 8-9 and must be performed in an amine-free buffer such as carbonate/bicarbonate.

In order to label BLG with FITC, a 2.5 mg/ml protein solution was prepared in 0.05 M borate buffer, pH 8.5. To 9.5 ml of this solution, 0.5 ml of 16 mg/ml FITC dissolved in DMF was added. After one night of incubation in the dark at 4°C, excess of unreacted dye was removed by ultrafiltration using a centrifugal ultrafiltration devices (Centricon cut-off 3000 Da). Labeled protein was stored in the dark at 4°C.

In order to calculate the protein concentration after the conjugation, the following equation was used:

$$\text{protein concentration (M)} = \frac{[A280 - (A494 \times 0.3)] \times \text{dilution factor}}{\varepsilon}$$

To calculate the amount of dye for each protein molecule the following equation was used:

$$\text{dye per protein molecule} = \frac{A494 \times \text{dilution factor}}{68{,}000 \times \text{protein concentration (M)}}$$

Emulsion preparation

Emulsions were prepared as follows: 0.5 g of soy oil (Sigma Co., St Louis, MO) were pre-emulsified with 9.5 ml of β-lactoglobulin solution (5 mg/ml in 0.05 M sodium phosphate buffer, pH 7) using a Vortex dispersing unit for approximately 1 min. The pre-emulsion was immediately homogenized in ice by using an ultrasound sonicator (MSE Soniprep 150) in 5 sonication cycles (1 minutes each) with a 14 microns amplitude intensity.

Polystyrene nanoparticles/β-lactoglobulin system preparation

For this purpose a BLG solution with a concentrations of 0.05 mg/ml was mixed with a constant concentration of NP (0.025 ml of original suspension/ml) in 0.05 M sodium phosphate buffer, pH 7. This was followed by an incubation of 60 minutes, in mild stirring conditions at room temperature, to promote interactions between the protein and NP.

Trypsin proteolysis

Limited proteolysis experiments were performed as follows: 0.01 ml of trypsin solution (TCPK treated, 1mg/ml in 0.025 sodium acetate, pH 4.5) were added to 1 ml of BLG solutions, “cream” and “serum” samples (in 0.05 M sodium phosphate, pH 7, final protein concentration: 1mg/ml) in order to reach a ratio protease/substrate of 1/100 w/w. Hydrolysis were carried out for 30 min at 37° C and stopped by addition of 0.02 ml of Soybean Kunitz Trypsin Inibitor (2 mg/ml in dd water).

For hydrolysis of nanoparticles bounded BLG (final protein concentration: 0.05 mg/ml) the same protocol was followed, adjusting the amount of protease in order to respect the protease/substrate ratio (1/100 w/w by addiction of 0.005 ml of a 0.1 mg/ml TCPK treated trypsin solution).

ELISA (enzyme-linked immunosorbent assay)

Enzyme-linked immunosorbent assays, also called ELISA, combine the specificity of antibodies with the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an enzyme (Hornbeck, 1991). They are usually carried out in 96-well microtiter plates. ELISA allows detection of antibody or antigen with considerable accuracy and sensitivity. In an ELISA both measurements of antigen and antibody concentration can be performed dependent on the design of the ELISA. There are two main variations on this method: an ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

An ELISA assay implies a five to six-step procedure:

1) Antigens or antibodies are absorbed/coated directly onto the plastic wells, 2) Plates are blocked to prevent further non-specific binding (optional), 3) Sample to be quantified (antibody or antigen) is added to the plate, 4) The bound analyte is detected by the addition of a specific antibody recognizing the antigen or antibody, being this antibody either conjugated to an enzyme or biotin-labeled, 5) Dependent on the previous step, either a substrate for the enzyme (to produce a colored product) or enzyme-labeled streptavidin (which specifically binds to biotin) is added. In this latter case, a suitable substrate is added, 6) The reaction is blocked (typically by adding acid or bases) and the amount of substrate/product in each well is quantified spectrophotometrically.

Between all the first 4 steps, wells are washed with buffer to remove unbound reagents and proteins. In our experimental approach, an ELISA tests were performed as competitive capture ELISA, by incubating specific mouse anti-BLG monoclonal antibodies (5G6 and 1E3, prepared in the same laboratory) with conjugates between NP and BLG. A 5 µg/ml BLG solution was prepared in carbonate buffer (14mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), and 0.1 ml of this solution were added to each well of a 96-well ELISA plate, and stored at 4℃ overnight for the coating step. 0.2 m l of each sample were loaded in the first lane of an uncoated ELISA plate (incubation plate). In particular, 0.1 mg/ml of BLG solution, BLG stabilized emulsions and BLG-nanoparticles were prepared. Buffer was also loaded as a negative control. Progressive 2-fold dilutions were then performed by mixing - in the next well – 0.1 ml material from each well with 0.1 ml washing buffer (PBS buffer, pH 7.4, 0.05% Tween 20). After adding 0.1 ml/well of specific mouse anti-BLG antibody (either 5G6 or 1E3 diluted 1:1000), the (incubation) plate was incubated for 1 hour at room temperature. After washing 3 times (with 0.2 ml of washing buffer) the previously BLG-coated plate, 0.1 ml samples (from each well of the incubation plate) were loaded to the BLG-coated plate, and incubation was carried out for one hour at room temperature. The plate was then washed extensively with washing buffer, and 0.1 ml of a horseradish peroxidase-conjugated secondary antibody (P260, DAKO A/S, Denmark), diluted 1:1000 in washing buffer, were added to each well, and the plate was incubated for 45 minutes at room temperature. After washing the plate, the enzyme substrate was prepared by mixing 2.8% (v/v) of 3,3',5,5'-tetramethylbenzidine stock solution (20 mM in 90%-10% v/v methanol/acetone) into an ELISA peroxide buffer (3.2 mM $NaBO_2H_2O_2 \bullet 3H_2O$, 40 mM CH_3COONa, pH 5.0). One hundred µl of the freshly prepared substrate were added to each well, and, after 10 minutes incubation, color development was stopped by adding 100

µl of 2M H_3PO_4 (the blue color turns into yellow). Absorbance was read by using a microplate reader (450 nm with a reference at 630 nm).

SDS-Page and Western-blotting

SDS-PAGE and Western-blotting were performed as follows: to 0.05 ml of each sample were added 0.05 ml of Laemmli denaturation buffer, boiled for 5 minutes and then loaded to a 12% acrylamide gel. Electrophoresis was carried out for 1.5 h at 16 mA for gel, using a Mini Protean 2 apparatus provided by Bio-Rad. After the electrophoretic run, gels were transferred for 1 h at 100 V on a nitrocellulose paper sheet (Protran, 0.45 µm, Millipore) using a Mini Trans Blot apparatus (Bio-Rad).

The nitrocellulose sheets were then incubated overnight in a blocking solution (0.5 % BSA in PBS). After an extensive washing, sheets were incubate with the primary antibodies solution for 2 hours (MAB 5G6 or 1E3 diluted 1:2000 in PBS), and then with the secondary antibodies solution (P260 polyclonal anti-mouse HRP, Dako, diluted 1:3000 in PBS) for 2 hours. Membranes were then developed using a developing solution made up of 0.6 mg/ml 4-chloro-1-naphtole, 10% methanol, 0.6% H_2O_2 in PBS.

Cell uptake study of BLG at emulsion interface

Cellular uptake of BLG at emulsion interface was studied on Mono Mac 6 cells (ACC124, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Mono Mac 6 (MM6) is a monocytic cell line derived from a 64-year old man with monocytic leukemia (Ziegler-Heitbrock *et al*., 1988). The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FBS), 1% penicillin and streptomycin (Gibco), 1% glutamine (Gibco), 2 mM non essential amino acids, 1mM sodium pyruvate and 9 µg/ml bovine insulin. Cells were grown at 37°C, and 5% CO_2 for three days, and were then used for experiments.

For this purpose cells were harvested, counted using a cell counter chamber and adjusted to a concentration of $1*10^6$ cells/ml. Vitality was assessed by Trypan Blue assay. Then, 150 µl of cell were added to single wells in a 96 wells-U-bottom plate and 25 µl of medium (RPMI 1640) were added to each single well. Also 15 µl of each compound (dissolved or diluted in DPBS) were added to the respective wells, to the respective time, as reported in the table below. Plates were incubated at 37° C with 5% CO_2. After the last addition, cells were spun down for 5 min at 1200 *x g,* resuspended in 150 µl cold FACS-wash buffer and spun down for 5 min at 1200 *r.p.m..* This passage was repeated 2 times.

Cells were then resuspended in 150 µl cold FACS-fixation buffer and the plate was analyzed by flow cytometry immediately or wrapped in tinfoil, kept at 4° C and analyzed later.

Sample tested in these experiment and their preparation were listed below:

- Free-BLG (BLG): lyophilized BLG was dissolved in DPBS buffer to a concentration of 1 mg/ml.
- FITC-BLG (F-BLG): FITC conjugated BLG was diluted with DPBS buffer from the stock solution (8.49 mg/ml) to a final concentration of 1 mg/ml.
- Emulsion FITC-BLG (E-BLG): Emulsion stabilized with FITC conjugated BLG was diluted from the stock solution (5 mg/ml) to a final concentration of 1 mg/ml in DPBS buffer.

Competition experiments were performed by addiction of free unlabeled BLG to the medium, in stoichiometric ratio 1:1 with FITC labeled protein or FITC-labeled-BLG stabilized emulsion.

Results

Determination of immunoreactivity of BLG adsorbed on hydrophobic interface

Various reports demonstrate that denaturation processes can alter the immunological properties of a protein. Hydrolysis (Selo *et al*., 1999, Iametti *et al*., 2002), heat treatment (de Luis *et al*, 2007), glycation (Corzo Martinez *et al*., 2010) are all events that may modify the intrinsic immuno-properties of a protein.

In order to determine the changes of immunoreactivity and the extent of this phenomenon, we developed an ELISA assay using two specific monoclonal antibodies (5G6 and 1E3, kindly provided by Prof. Hanne Frøkiær from University of Copenhagen) , that is suitable for detecting changes in immunoreactivity after adsorption of BLG on two model hydrophobic surface.

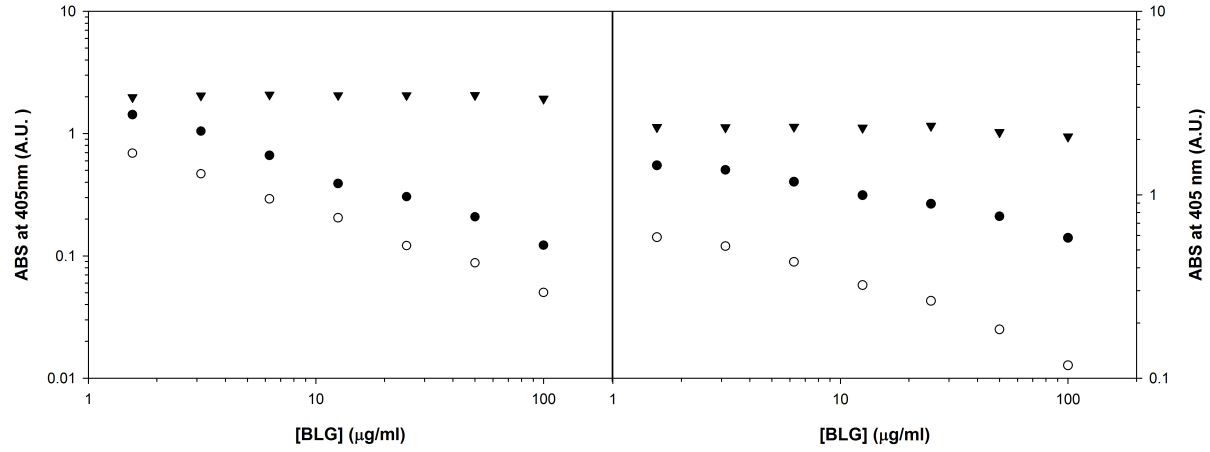

Fig 4.1: *ELISA inhibition curves for free BLG (●) and BLG stabilized emulsion (○). BSA (▲) was used as negative control. Proteins in each samples are present in the same concentration. 5G6 monoclonal antibodies (right panel) and 1E3 monoclonal antibodies were used.*

The Figure 4.1 shows the inhibition curves in ELISA experiments carried out with BLG stabilized emulsions samples. Using both antibodies, it seems that protein present at the water/oil interface is more reactive than the one present free in solution. The increase in immunoreactivity is between 30% for the monoclonal antibody 5G6 and 120% using antibody 1E3.

This increase in immunoreactivity may be due to either an increase in the number of interfacial epitopes exposed after denaturation of the protein or to a sort of structure

locking that stabilize the epitopes that are already present. This last hypothesis seems to be more creditable for the explanation of the phenomenon, since the exposure of new binding sites for the antibody would vary affinity constant of the system for the antibodies, which would result in a different slope of the inhibition curve. This “apparent” increase in the protein concentration can be explained by stabilization of the structure in a single structure, which is no longer in equilibrium with non-reactive forms naturally present if the protein is free in solution. In fact, the BLG, when present in solution, is present in a monomer-dimer equilibrium. A work made in the professor Frøkiær lab (data not published) report that the dimeric form appears to be far more immunoreactive than the monomer. The interfacial effect could block the protein structure in a form more similar to the dimmer of the BLG.

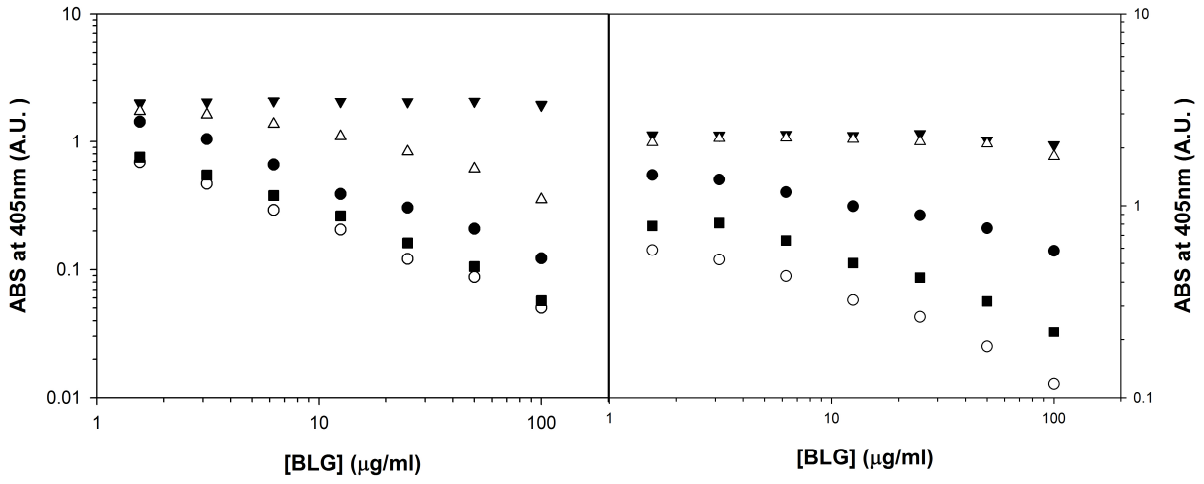

Fig 4.2: *ELISA inhibition curves for free BLG (●), BLG stabilized emulsion (○), trypsin hydrolyzed emulsions (■) and BLG hydrolyzed by trypsin in solution (Δ). BSA (▲) was used as negative control. Proteins in each samples are present in the same concentration. 5G6 monoclonal antibodies (right panel) and 1E3 monoclonal antibodies were used.*

It is also relevant to note that the bound protein, after hydrolytic action by trypsin appears to be more immunoreactive then the hydrolyzed protein in solution, that decreases its reactivity by 50% (Figure 4.2). This behavior is found using both antibodies. A possible explanation can be attributed to the presence of larger peptides after hydrolysis at the oil/water interface. These species keep intact their immunoreactivity. Also, another possible explanation may due to the increased reactivity of intact protein on the surface of oil droplets, which raise the responsiveness of the system.

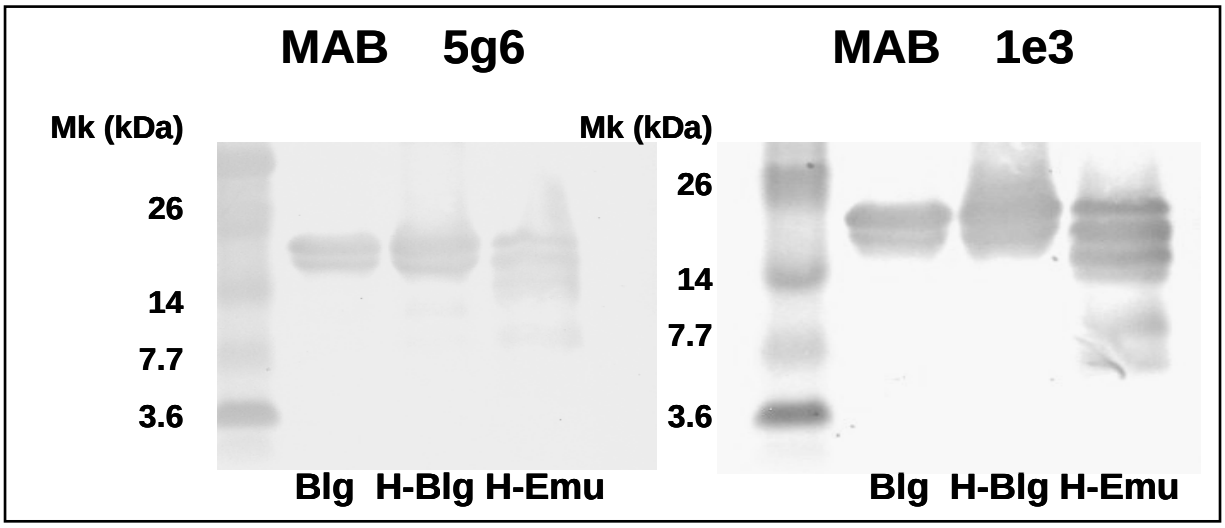

Fig 4.3: *western blot analysis of native BLG (Blg), trypsin hydrolyzed BLG in solution (H-Blg) and BLG hydrolyzed with trypsin on emulsion interface (H-Emu). Western-blotting membranes were developed using 5G6 monoclonal antibodies (right panel) and 1E3 monoclonal antibodies (left panel).*

In order to highlight the molecular determinants that allow to maintain of the immunoreactivity after hydrolysis with trypsin, Western Blotting analysis was performed using the same monoclonal antibodies used in the ELISA assays as primary antibodies. It is evident that in samples hydrolyzed at the oil/water interface (Figure 4.3) high molecular weight (8000-6000 Da) and immunoreactive peptides are present. These peptide seem to be not present in the samples hydrolyzed in solution. It is also interesting to note how the same peptides react to both antibodies.

Also changing the physical chemical nature of the hydrophobic material, the reactivity of BLG bound to latex nanoparticle remains greater than that of the protein free in solution (Figure 4.4). Even in this case protein seems to be more immunoreactive after adsorption if using 1E3 antibody compared to antibody 5G6. The hypothesis of the stabilization of the protein structure, with a more stable reactive epitope exposure seems to be further supported by these experimental data.

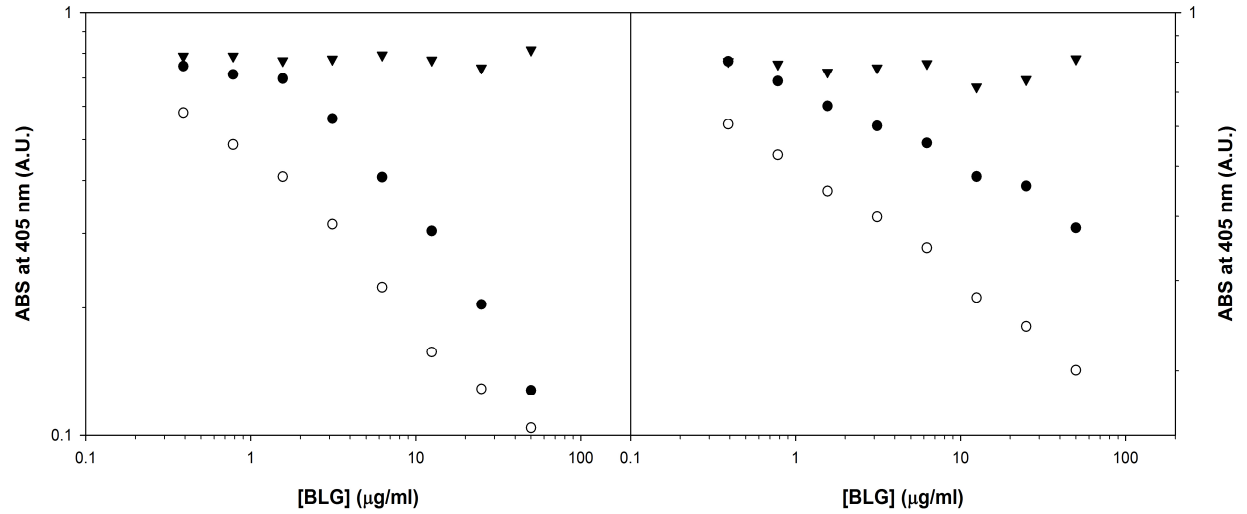

Fig 4.4: *ELISA inhibition curves for free BLG (●) and BLG adsorbed on latex nanoparticles. BSA (▲) was used as negative control. Proteins in each samples are present in the same concentration. 5G6 monoclonal antibodies (right panel) and 1E3 monoclonal antibodies were used.*

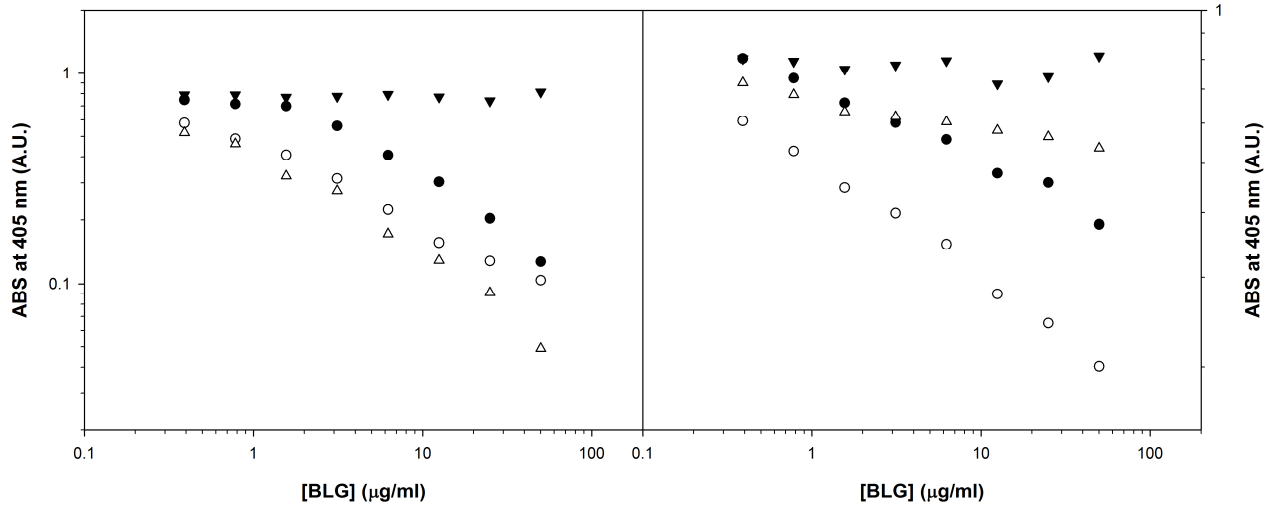

Fig 4.5: *ELISA inhibition curves for free BLG (●), BLG adsorbed on latex nanoparticles (○), trypsin hydrolyzed BLG on latex nanoparticles (■) and BLG hydrolyzed by trypsin in solution (Δ). BSA (▲) was used as negative control. Proteins in each samples are present in the same concentration. 5G6 monoclonal antibodies (right panel) and 1E3 monoclonal antibodies were used.*

The immunoreactivity of the protein after hydrolysis remains unchanged, and unexpectedly it increased after hydrolysis even after adsorption on latex nanoparticles (Figure 4.5). The degree of immunoreactivity of the peptides released after hydrolysis on the nanoparticles can be attributed to the different molecular nature of them respect of the peptide released after BLG hydrolysis on oil droplets or from BLG free in solution.

It was already demonstrated in the previous chapter that the tryptic peptides derived from hydrolysis of the nanoparticles are significantly different from those resulting from hydrolysis of oil/water interface. In addition, the peptides coming from hydrolysis of BLG on the surface of nanoparticles are accounted by their “not tryptic” nature, because many of them do not seem to have been hydrolyzed in correspondence of to lysine or arginine, but on other residues. This behavior may explain, at least in part, differences in immunoreactivity of these samples

Cell uptake of BLG at emulsion interface

In order gather information about the implications of the interfacial proteins denaturation in biological systems, we have carried out a few preliminary experiments on BLG uptake by human monocytes. More precisely, absorption tests were conducted using free BLG in solution or presented to the cells at the oil/water interface.

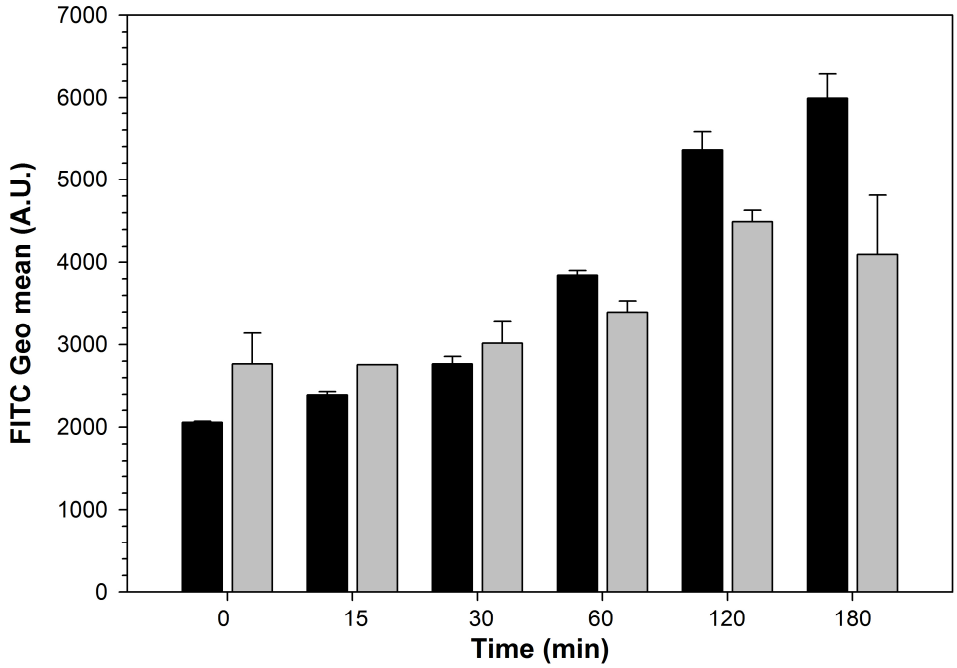

Fig 4.6: *absorption kinetics by monocytes of free BLG solution (black bars) and BLG on oil nanodroplets (gray bars.) Fluorescence intensity was measured by flow citometry.*

Figure 4.6 shows the monocytes absorption kinetics of BLG in solution (black bars) and of BLG adsorbed on the surface of nano-emulsions (gray bars). Both proteins are present in the assay at a concentration of 0.08 mg/ml.

As evident in the Figure the absorption by monocytes appears to be significant only after 30 min of incubation, reaching a maximum of absorption after 3 hours of incubation. The absorption of BLG adsorbed at the oil/water interface seems to be higher at low incubation times. This phenomenon could be due to a nonspecific adsorption of protein on the cell surface. The first significant increase in fluorescence due to absorption of the protein by monocytes occurs after two hours. After three hours of there is no further a significant overall increase in fluorescence of the cells. At maximum incubation time the uptake of the protein free in solution by cells is greater than the protein adsorbed on the nano-emulsions, allowing us to hypothesize two different mechanisms of internalization of BLG. Figure 4.7 shows the absorption kinetics of BLG free in solution and adsorbed on the surface of oil nanodrops. In this experiment, the samples were diluted in DPBS buffer and stored at 4° C for 24 h. The high ionic strengt h of the medium leads to changes in the structure of the emulsion with the appearance of coalescence phenomena and consequent migration phenomena at the interface of the protein solution.

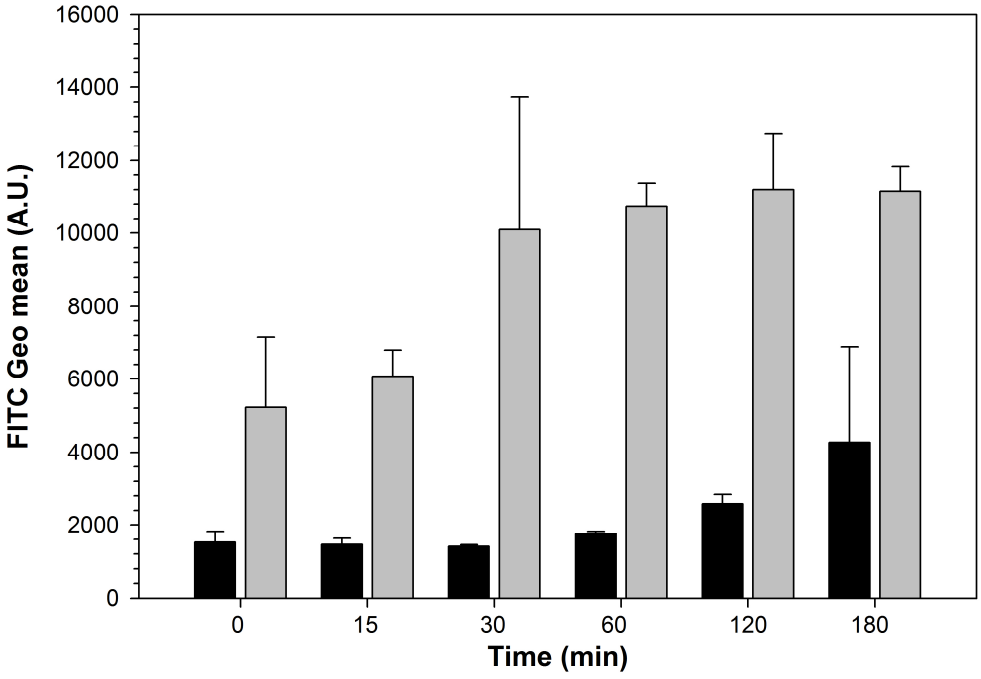

Fig 4.7: *absorption kinetics by monocytes of free BLG solution (black bars) and BLG on oil nanodroplets (gray bars). Both samples were kept at 4° C for 24 h in DBPS buffer prior addiction to the cells. Fluorescence intensity was measured by flow citometry.*

As shown in the Figure, the absorption kinetics of the protein in solution are comparable with the kinetics of the previous experiment, while uptake of the protein adsorbed on the surface of the emulsion is greatly increased. This phenomenon could be attributed to the changing of chemical and physical conditions of the emulsion after storage for 24 h in high ionic strength environment that is recognized to be highly disruptive for these systems. The maximum absorption peak is achieved already after 30 minutes of incubation, with no more further significant increases in fluorescence longer times of incubation. We still need further experiments to clarify the dynamics that govern this phenomenon.

In order to clarify the aspects related to the protein uptake mechanisms by monocytes we carried out competition experiments. These experiment are characterized by the presence of stoichiometric amount of unlabeled free protein.

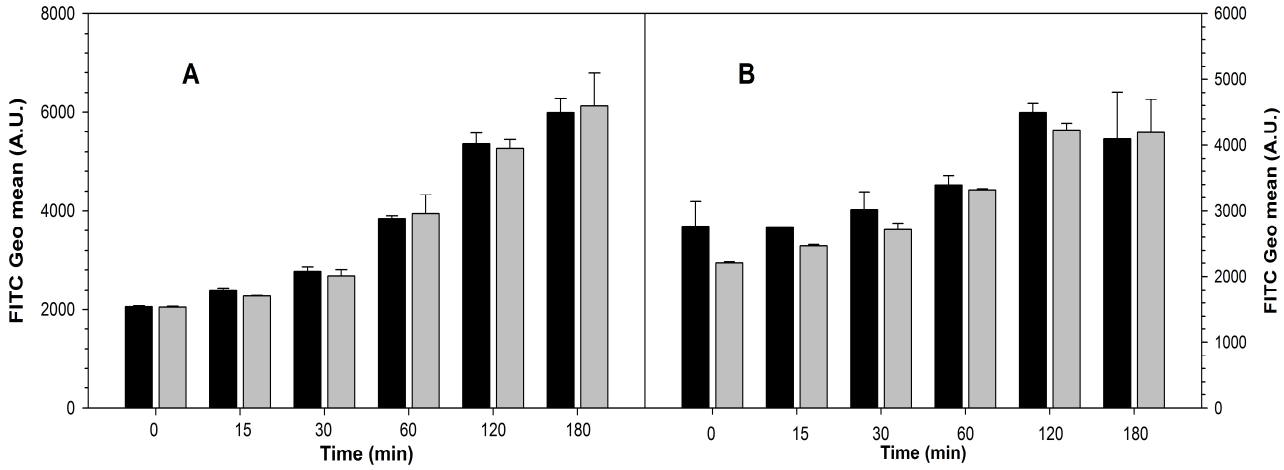

Fig 4.8: *competition absorption kinetics by monocytes of free BLG in solution (Panel A) and adsorbed on oil nanodroples (Panel B). Black bars show absorption kinetic of samples with protein concentration of 0.08 mg/ml of FITC-labeled BLG. Gray bars show absorption kinetic of samples with protein concentration of 0.08 mg/ml of FITC-labeled BLG wit addiction of 0.08 mg/ml of free unlabeled BLG. Fluorescence intensity was measured by flow citometry.*

As evident in Figure 4.8/A stoichiometric additions of unlabeled BLG to the system does not appear to alter significantly the absorption of the protein. Even a total concentrations of BLG around 0.16 mg/ml does not seem to be sufficient to saturate the system, and comparable concentration of fluorescent BLG can be found within the cell regardless of the presence of the unlabeled competitor. We do not expect in this frame a

higher affinity of the cell for the protein labeled with fluorescein compared to the unlabeled protein.

The same competition experiment was carried out by adding stoichiometric concentrations of unlabelled BLG free samples of emulsion stabilized by BLG. In Figure 4.8/B shows the results of the test. In this case, the absorbtion of labeled BLG present on the oil droplets surface seems to be altered slightly at low incubation times. Significant differences are visible up to 30 min of incubation. Starting from 60 min of incubation, no significant differences are observable.

Conclusions

In conclusions this work provides information about the changes in immunoreactivity of BLG after adsorption on two model hydrophobic interfaces. ELISA experiments demonstred that BLG increases its immunoreactivity after interaction with both liquid/liquid and solid/liquid interfaces. This behavior should be explained by the locking in a more reactive conformation, and not by the exposure of novel epitopes. Moreover, after hydrolysis with trypsin, the overall reactivity remains high compared with the protein hydrolyzed in solution. This high reactivity of the hydrolyzed samples was explained by Western-blot experiments, which shown the presence of high molecular mass peptides in the hydrolytic products of surface-bound BLG, that are absent in the protein hydrolyzed in solution. These high molecular mass peptides remain reactive against the monoclonal antibodies used in this study.

Another relevant aspect is how the BLG internalization kinetics by monocytes adsorbed on the oil nanodroplets is different compared with the free BLG. In fact, it seems that free BLG is absorbed more efficiently and rapidly than BLG adsorbed on an emulsion interface. But, if using “ripened emulsions” the behavior seems to be opposite, with a high absorption efficiency by monocytes of the larger particles originate after “aging” the original emulsion under condition of high ionic strength.

Competition experiments, carried out by adding free unlabeled protein to both free and emulsion BLG systems, shows how the high absorption efficiency of monocytes. No relevant differences were observed in our experiments. Whether all these evidence may involve the existence of independent pattern for intracellular uptake of free and bound BLG (Marengo *et al.*, 2011) remains to be verified.

These result pointed out the importance of protein physical state on its physiological outcome. We are actually performing studies aimed to discover and describe more in deep this important aspect, with relevant implication in both immunological and food science.

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Chapter 5)

β-LACTOGLOBULIN UNFOLDING AFTER ADSORBTION ON POLYSTYRENE SURAFCE: AN "*in silico*" SIMULATION

Introduction

The physiological properties of molecules in general, and of a protein in specific, relies on their behavior at the molecular scale under a number of circumstances, this way in turn by is affected by their surface interactions in the physiological medium of interest. A central issue for the *in vivo* action of a bioactive protein (an allergen, an antibacteric peptide..) is their surface behavior in terms of interaction with other molecules and surface present in the same environment (Kasermo 2002, Ratner *et al.* 2004). The interaction of bio- and nano-materials with proteins is therefore extremely important both for their performance and the possible biohazards, and the atomic scale modeling of protein adsorption on surfaces may shed light on many issues concerning their performance in fields ranging from nanomedicine to drug delivery, and from implant biotechnology to environmental issues.

From a more basic viewpoint, a similar approach can be adopted to model other non-covalent interactions, such as molecular recognition phenomena (Raffaini *et al.*, 2008) that are relevant in enantiomeric drug separations and for developing of nanosized drug vectors.

Theoretical modeling of these phenomena can now be carried out with molecular simulations due to the availability of increasingly sophisticated algorithms and programs, but mostly due to increasingly larger computing capacity in terms of speed and data storage. Molecular simulations, together with the modeling of theoretical frames, have been widely used to study the behavior of polymeric systems, focusing in particular on their large-scale properties through coarse-grained models to calculate the molecular size, transport, and rheological properties. However, these models cannot fully account for the protein structure used for molecular recognition phenomena, where the atomistic details are most relevant. In recent years, some authors shown that atomistic computer simulations can be usefully employed to model biomaterials and their surface properties, in particular protein adsorption (Ganazzoli 2005, Raffaini *et al.*, 2007).

This approach can provide significant new insights into the behavior of interactions between proteins hydrophobic interfaces in a physiological environment, giving atomistic information about protein denaturation and surface spreading (or, possibly, ordering). In particular, atomistic simulations can account for the protein secondary structure (α-helices or β-sheets), and for the surface of different materials with dissimilar wettability. These conditions can be very important for predicting protein structure reorganization in a

“foreign” envinroment. “Soft” proteins such as albumins undergo extensive surface rearrangements, whereas “hard” proteins such as lysozyme are more resistant to major changes, at least on hydrophilic surfaces. Additionally, the surface ordering of proteins on graphite (Raffaini 2004) may lead to refolding to a new secondary structure (Zhdanov *et al.* 2001, Castells *et al.* 2005), or yield a spontaneous nanopatterning (Svaldo-Lanero *et al.* 2008) that can be used as a template for inducing further supra-molecular ordering. Early theoretical approaches to study protein adsorption on foreign surfaces were based on semi-macroscopic colloidal models, which proved quite satisfactory for rigid proteins on a charged surface (Roth *et al.* 1995).

Proteins can be viewed as copolymers formed by a specific sequence of the natural aminoacids, and their simplest theoretical description is in terms of amphiphilic copolymers in a selective solvent (Dill *et al.* 1995, Onuchic *et al.* 1997, Ganazzoli 1998, Ganazzoli 2000). This approach has provided some clues about protein folding and the kinetics of surface absorption (Zhdanov *et al.* 2001, Castells *et al.* 2002), but neglected all atomistic features, including the full protein structure. Currently, such features and the detailed pattern of the outer electrostatic potential cannot be ignored any longer. To overcome these limitations, some works were aimed at modeling protein adsorption on the surface of bio- and nano-materials through forcefield-based atomistic simulations. The chosen methodology involves energy minimization (molecular mechanics, or MM) and molecular dynamics (MD) simulations at a given temperature. This procedure allows for the possible surface spreading considering both an implicit solvent through an effective dielectric medium and explicit water.

Such an approach can in principle provide a thorough picture of the adsorbtion process at the nanometer scale. Therefore, the surface hydration of biomaterials can first be investigated (Raffaini 2007, Raffaini *et al*., 2010), and then protein adsorption can be modeled using a common methodology with a general simulation protocol. Protein subdomains with different secondary structures and hydropathy and lysozyme were studied on the surfaces of biomaterials with dissimilar wettability, so that the surface-induced conformational rearrangements and the nanostructure of the adsorbates could be analyzed, together with the energetic of the process.

Bioinformatics is a branch of computational science that exploits the computer calculus and graphic visualization potentiality in order to study biological, chemistry and physics issues. Researches in life science is generating a massive amount of data, that they cannot managed and analyzed without the use of computers. Information technology

can be used to collect, to organize, to analyze and to store this kind of data. Analysis of genetics or proteins sequences, analysis of protein three-dimensional structures, simulations of molecular dynamics is some of the opportunity offered by bioinformatics.

Molecular modeling (MD) is a general word that covers a wide selection of computational and graphical techniques, aimed to calculate, build, simulate and analyze molecular structures, as well as to calculate chemicals and physical properties. The knowledge of the three dimensional structure of a protein is important to plan *in silico* experiments aimed to discover the mechanics that governs its folding-unfolding behavior.

Programs for molecular modeling are graphics interfaces used to develop models, to defines force fields and control the simulation engines. The calculus that governs a simulation based on *force fields* is aimed at computing and estimating the potential energy of a configurations of atoms. The computation of this energy, and its first and second derivates as function of atoms coordinates, gives essential information for minimization, for harmonic vibrational analysis and for dynamics simulation. Computations are made by a simulation engine, that is a program based on force field. Simulation engines are computational suites developed to manage the force field application during energy minimization, molecular dynamics, and other molecular mechanics simulation.

Force field is defined as the potential energy function and by the entire set of parameters required to define a potential energy surface. It is fundamental to remember that the force field is the only approximation not ignorable in molecular modeling. The quality of a force field, its applicability to the case under investigation exam and its capacity to compute particular properties measured during the simulation determine the results validity. Force fields can be classified as follow:

- Force fields based on general rules, applicable to a broad range of elements of the periodic tables
- Classic force field (first generation force field), but suitable to address biochemical problems

Aim of the study

The aim of this study is to create a *in silico* reliable model for β-lactoglobulin structural changes after adsorption on a hydrophobic polystyrene surface, and to compare results obtained with this computational approaches with the experimental data exposed in the previous chapters.

Materials and Method

Experimental *in silico* simulation were performed in the computation facilities of Dt. Ivano Eberini, at the Pharmacological Science Department of the University of Milan. All the computational procedures were carried out by using Molecular Operating Environment (MOE), an interactive, windows-based chemical computing and molecular tool with a broad array of scientific applications.

Building polystyrene surface

Polystyrene surface was built using the polymer builder program of the “build module”. The repeat unit (styrene monomer) is already available and optimized in the MOE database. We prepared a single chain of 140 styrene monomers in syndiotactic arrangement. This chain was replicated 16 times in order to obtain a single layer of latex of approx. 16.0 X 30.5 nm. This layer was replicated to produce a double layer.

In order to produce a perfectly plain polystyrene bilayer and to carry out a MD simulation with an implicit solvent model we applied a wall restraint according to the fundamental function form

$$p(t) = \begin{cases} 0 & \text{if } t < 0 \\ t^3(6 - 8t + 3t^2) & \text{if } t \text{ in } [0, 1] \\ t & \text{if } t > 1 \end{cases}$$

which is used with t set to various values to achieve the restraint conditions. The function p is twice continuously differentiable since p (0) = 0, p (1) = 1 and p (0) = p (1) = 0.

The wall restraint enclosed all the polystyrene chains inside the box; the pool were restrained in an axis aligned box were d defines the half box dimensions of each axis. For an atom with coordinates (x,y,z) the box restrain energy E is given by:

$$E_w = W[p(x-x_0-D_x) + p(x_0-D_x-x) + p(y-y_0-D_y) + p(z-z_0-D_z) + p(z_0-D_z-z)]$$

The MMFF94x force field was used with a generalized Born-implicit solvent model. After an energy minimization step down to a root-mean-square of 10^{-5}kcal $mol^{-1}Å^{-1}$, the bilayer structural properties were carefully checked.

BLG preparation

The B variant of BLG crystallized by *Qin et al*. (1998), deposited in RCSB as 1BSQ, was prepared, optimized and used in BLG/polystyrene MD simulation. The crystallization water molecules were removed and hydrogens were added. The energy of the protein was minimized down to a root-mean-square of 10^{-5}kcal $mol^{-1}A^{-1}$.

BLG/polystyrene preparation

BLG was positioned on the polystyrene double layer with a potential energy continuous monitoring system. We drew up the BLG structure to the polystyrene bilayer down to an energy minimum. This procedure was repeated for other five different spatial orientation of BLG, in order to test different interaction fashions.

MD simulation

Five different MD simulations were carried out according the following protocol: 100 ps heating from 0 to 300 K, 100 ps equilibration at 300 K, 500 ps production. The light bonds were constrained. The integration algorithmic was Nose-Poincare-Andersen hamiltonian equation of motion, which is able to generate true ensemble trajectories. The MD simulation with the highest RMSD was run for 5 ns.

Results

The *in silico* analysis of the BLG denaturation process after adsorption on a hydrophobic surface - in our case polystyrene - has highlighted a number of interesting features that lead a more or less extensive protein destructuration which depend on several parameters.

The nature and amplitude of the structural changes is a function of the orientation in which the protein is interacting with the hydrophobic matrix. As evident in Figure 5.1, the RMSD, parameter that describe the extent of the displacement of a specific residue, or, if extended to the whole protein molecule, the sum of the displacements of all residues, appears to be significant only for the position defined as “5”, where the molecule is posed in contact with the latex surface with the surface placed below the small alpha helix (Figure 5.2).

RMSD = 2.971 A

	1	2	3	4	5	6
1: 2_inizio.pdb.A	0.00	1.34	2.08	1.77	4.29	1.72
2: 2_fine.pdb.A	1.34	0.00	2.18	2.10	4.23	1.68
3: 3_fine.pdb.A	2.08	2.18	0.00	2.41	4.62	2.28
4: 4_fine.pdb.A	1.77	2.10	2.41	0.00	4.20	1.95
5: 5_fine.pdb.A	4.29	4.23	4.62	4.20	0.00	4.26
6: 6_fine.pdb.A	1.72	1.68	2.28	1.95	4.26	0.00

4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

Fig 5.1: *RMSD values of 5 different MD of BLG adsorption on polystyrene nanoparticles*

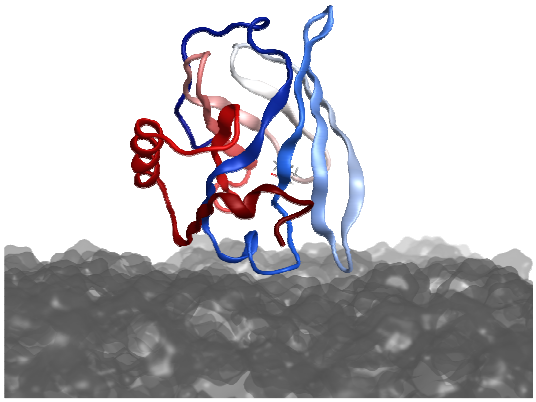

Fig 5.2: *BLG orientation on polystyrene surface in the simulation number 5*

In this orientation, the protein structure is extensively altered with respect to the native tertiary structure, and also its secondary structure undergoes extensive rearrangements (Figure 5.3, Table 5.1).

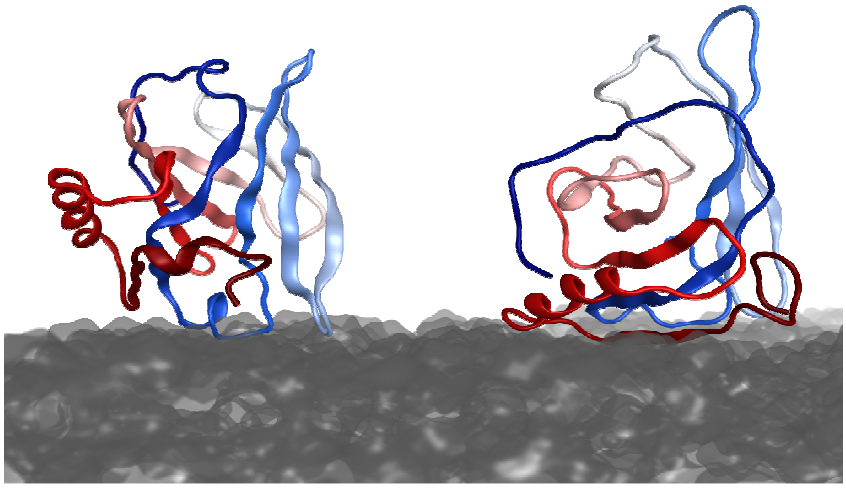

Fig 5.3: *comparison of BLG structure before the start (structure on left) and after the end (structure on right) of the MD*

When other orientation were tested only some unfolding nuclei of the tertiary structure could be detected, but no significant changes of secondary structure were evident (Table 5.1). Estimating the percentage of secondary structure were performed using the program STAN (The Structure Analyser servers - xray.bmc.uu.se / cgi-bin / gerard / rama_server.pl).

Pose	% α-helix	% β-sheet	Pose	% α-helix	% β-sheet
2_start	17.901	43.210	**5_ start**	15.432	42.593
2_end	16.667	43.827	***5_ end***	8.025	47.531
3_ start	16.049	42.593	**6_ start**	15.432	43.210
3_ end	11.111	35.185	***6_ end***	12.346	43.827
4_ start	17.901	39.506			
4_ end	14.815	40.741			

Tab 5.1: *secondary structure percentage of BLG adsorbed on polystyrene NP before and after MD. Samples differ as for orientation of the interacting surface. Percentages of secondary structure were computed by using the program STAN (The Structure Analyser servers - xray.bmc.uu.se / cgi-bin / gerard / rama_server.pl)*

Turning to the analysis of the structure of BLG after adsorption on the latex interface in orientation "5" (Figure 5.4) is well evident how the structure is dramatically open, compared with the native structure, in the area between the alpha helix and the beta-barrel. In this area there is the Cys_{121} residue, which is thus fully exposed to the solvent, as previously demonstrated experimentally in chapter 3. In addition, this particular destructuration shifts both Trp away from their natural neighboring quenchers supporting the spectrofluorimetrical findings (and their interpretations) as presented in chapter 3. It is also interesting to note that the beta-barrel almost completely loses its structure with the concomitant exposure to the solvent of "new" hydrophobic patches.

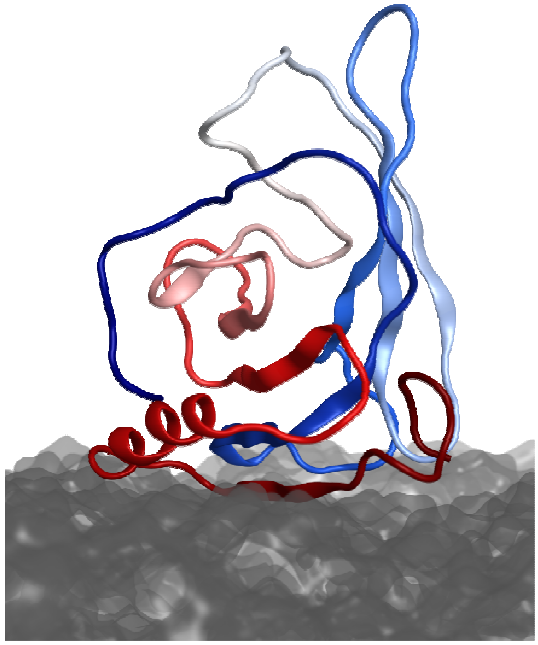

Fig 5.4: *BLG structure on polystyrene surface in the MD of the pose number 5*

Not only the tertiary structure is affected after the interaction with the hydrophobic surface. The alpha-helix content decreased from 15.5 % to 8 %, whereas the beta sheet structures grew from 42.5 % to 47.5%. On the basis of these results we can include BLG into the category of the "hard" protein, because it reorganizes its structure, but it still maintains one.

The mechanism that naturally orientates the protein on the hydrophobic surface remains yet unknown, and we have not information whether BLG adheres to the styrene surface with random orientation. Moreover, this kind of simulation does not take into

account the structural changes that may happen before the protein interacts with the hydrophobic surface.

Unfortunately, at the date of submission of this work the calculations of total energies of various systems with the protein oriented in 5 different poses are not yet finished. The calculation of these energies are important to evaluate which is the preferential adsorption position.

Conclusions

In this work we used an *in silico* approach to study the unfolding process ensuing from adsorption on a hydrophobic surface. We demonstrate that the orientation in which the protein is posed on the latex surface has a huge influence on the final structure after the MD run. In the pose number “5”, we obtained a structure compatible with the experimental result shown in chapter 3. We also proved that BLG do not lose completely its structure, but it reorganizes the native ones to a more energetically favorable one. The overall energy of the 5 different systems will be data available soon, and will confirm if pose “5” is the more energetically favorable and consequently, the more likely to be present on the latex interface. Future prospective will implies the study of the protein migration from the solution to the surface.

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General conclusions

In this PhD thesis I have studied in detail several aspects of the structural and biological implications of of proteins adsorption on hydrophobic interfaces. The spontaneous adsorption of protein molecules on interfaces is a ubiquitous phenomenon in natural and man-made systems. This phenomenon plays a central role in many fields, such as health, food, environmental science, and biochemical or immunochemical analysis. The structural rearrangement caused by the direct contact with the sorbent phase may affect protein biological activity, including allergenicity, bioavailability, and ability to bind micro- and macromolecular ligands. Whether proteins unfold randomly or through subsequent ordered and eventually reversible steps remains often unknown, and information about the molecular determinants of the “gain of function” or the “loss of function” observed upon adsorption is scarce.

For this purpose I have used as proteins model molecules with high interest for the food science and pharmacological fields, and for their application to nanotechnology. In particular I studied soy proteins (β-conglycinin and glycinin) and bovine whey protein (β-lactoglobulin).

Regarding soy proteins, I found that β-conglycinin undergoes a structural rearrangment at the oil-water interfaces. In fact, the fluorescence spectra of the protein in β-conglycinin stabilized emulsion are red-shifted compared with the fluorescence spectra of the native protein. In particular, β-conglycinin tryptophans seem to increase their exposure to solvent water when the protein interacts with the oil surface. Tryptophans in the mature form of β-conglycinin are present only in the in the N-terminal extension regions, the least hydrophobic areas, of α and α’ subunits. After emulsion digestion with trypsin, some peptides were released into the aqueous phase, including the tryptophan containing regions in the extension domains. Large peptides from the core region are released as well. These peptides come from the least hydrophobic regions of this domain.

In conclusion, it is possible to hypothesize that the core regions of the β-conglycinin subunits interact with the oil phase, whereas the extension regions of the α and α’subunits protrude in the aqueous medium. Our proteolysis data also suggest that the core domain is oriented with its least hydrophobic regions exposed to the water.

BLG studies demonstrate that also this protein undergoes an extend structural rearrangement. In fact, the intrinsic fluorescence spectrum of adsorbed BLG is red-shifted

compared with the free-protein one thus indicating that the adsorbed protein assumes a new structure in which Trp_{19}, usually buried inside the hydrophobic core, is exposed to water. Moreover, adsorbed Blg increases ≈2 folds its global quantum yield. This phenomenon could be explained either by the moving of Trp_{61} away from the Cys_{66}-Cys_{160} disulphide bond, and/or by the moving of Trp_{19} from Arg_{124}, thus removing fluorescence-quenching interactions within the protein structure. The only free thiol in BLG is on Cys_{121}, which is buried in the native structure, but becomes readily and almost completely accessible after adsorption. The overall Blg surface hydrophobicity seems to increase after interaction with the hydrophobic surface, confirming the occurrence of major rearrangements. The adsorbed protein is resistant to proteolysis by trypsin where the free protein is almost completely digested in the same conditions. All these data demonstrate an extended stretch of the native structure after adsorption on hydrophobic surfaces with the exposure of new protein regions usually buried from the aqueous media.

Immunoreactivity of BLG is markedly altered upon absorption. The amplitude of the observed differences is also depending on the nature of the sorbent material.

Another relevant aspect is how the BLG internalization kinetics by monocytes adsorbed on the oil nanodroplets is different compared with the free BLG. In fact, it seems that free BLG is absorbed more efficiently and rapidly than BLG adsorbed on an emulsion interface. But, if using “ripened emulsions” the behavior seems to be opposite, with high absorption efficiency by monocytes of the larger particles originate after “aging” the original emulsion under condition of high ionic strength. Competition experiments, carried out by adding free unlabeled protein to both free and emulsion BLG systems, shows how the high absorption efficiency of monocytes. No relevant differences were observed in our experiments. Whether all these evidence may involve the existence of independent pattern for intracellular uptake of free and bound BLG remains to be verified.

In silico denaturation experiments confirm our experimental results, and they given evidence of the importance of the protein molecule orientation for the final rearranged structure.

In conclusion, this thesis describes in deep a number of aspects regarding protein adsorption on hydrophobic interfaces. Future prospective will be addressed to evaluate the *in vivo* consequences of this phenomenon. Moreover, the “folding” properties of these structures will be be studied, in order to develop man-made “chaperon-like” nanotools.

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Milano 10/01/2012

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Scientific production

Articles

▪ M. Miriani, M.Keerati-u-rai, M. Corredig, S. Iametti, F. Bonomi
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Denaturation of soy proteins in solution and at the oil–water interface: A fluorescence study

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ABSTRACT

Structural changes ensuing from denaturation of soy proteins in solution or occurring at the oil–water interface were studied by fluorescence spectroscopy. Studies were carried out on solutions and emulsions stabilized with β-conglycinin or glycinin. Tryptophan fluorescence spectroscopy was used to evaluate tertiary structural changes. The binding of fluorescent dyes and the accessibility of reactive cysteine thiols were also used to better identify structural changes of these proteins in solution. Protein conformational changes after interaction with the hydrophobic oil surface were compared with those ensuing from physical (temperature) or chemical denaturation (chaotropes). Results from solution denaturation experiments indicate that structural changes of β-conglycinin by both temperature and chaotropes are reversible under appropriate conditions, and result in a rearrangement of the supramacromolecular assembly of the protein structure. On the other hand, glycinin treated under the same conditions undergoes irreversible denaturation in solution at temperatures well below 90 °C. Both proteins undergo partial denaturation after adsorption on the lipid surface, and no further denaturation occurs upon heating of the emulsions prepared with either protein.

▪ B. Huschka, F. Bonomi, M. Marengo, M. Miriani, K. Seetharaman
"Comparison of lipid effects on structural features of hard and soft wheat flour proteins assessed by front-face fluorescence" **2011,** Food Chemistry, *in press*

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Comparison of lipid effects on structural features of hard and soft wheat flour proteins assessed by front-face fluorescence

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ABSTRACT

Front-face fluorescence was used to assess changes in the structural features of proteins in wheat flour dough upon addition of different exogenous lipids. Structural changes resulting from the solvation of proteins and from the mechanical deformation of proteins in dough or dough containing lipids were measured. The effects of lipid type and addition on changes in overall protein surface hydrophobicity were estimated by titrating soft and hard wheat flours, mixed with water and varying the lipid levels, with increasing concentrations of the fluorescence hydrophobic probes 1,8 anilino-napthalene-sulphonate (ANS) and thioflavin T. The lipid type and level modified the exposure of the probe to the solvent. The effects of lipids were more apparent with soft wheat flour having low-affinity hydrophobic sites on the protein surface. The dough was then characterized upon consistent mixing and physical modification in the farinograph at constant water and ANS/thioflavin T concentration, while varying the type and amount of lipids. Lipid-dependent shifts toward longer wavelengths in the probe fluorescent emission with low-protein flour suggest differences in protein coating effects related to lipid structure and protein quality.

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Structural changes of soy proteins at the oil–water interface studied by fluorescence spectroscopy

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ABSTRACT

Fluorescence spectroscopy was used to acquire information on the structural changes of proteins at the oil/water interface in emulsions prepared by using soy protein isolate, glycinin, and β-conglycinin rich fractions. Spectral changes occurring from differences in the exposure of tryptophan residues to the solvent were evaluated with respect to spectra of native, urea-denatured, and heat treated proteins. The fluorescence emission maxima of the emulsions showed a red shift with respect to those of native proteins, indicating that the tryptophan residues moved toward a more hydrophilic environment after adsorption at the interface. The heat-induced irreversible transitions were investigated using microcalorimetry. Fluorescence spectroscopy studies indicated that while the protein in solution underwent irreversible structural changes with heating at 75 and 95 °C for 15 min, the interface-adsorbed proteins showed very little temperature-induced rearrangements. The smallest structural changes were observed in soy protein isolate, probably because of the higher extent of protein–protein interactions in this material, as compared to the β-conglycinin and to the glycinin fractions. This work brings new evidence of structural changes of soy proteins upon adsorption at the oil water interface, and provides some insights on the possible protein exchange events that may occur between adsorbed and unadsorbed proteins in the presence of oil droplets.

Congress oral presentations/Presentazioni orali a congressi

▪ M. Miriani, S. Iametti, F. Bonomi, M. Marengo, R. Hempelmann, S. Perego, A. Ferraretto
Contribution title: "Food proteins conjugates to magnetic nanoparticles: evaluation of their cellular toxicity"
Congress:Componenti nutraceutici della dieta: aspetti biochimici e tossicologici
Date: 12nd June 2009, Rimini

▪ M. Miriani, M.Keerati-u-rai, M. Corredig, F. Bonomi
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Congress abstract contributions/Abstract a congressi

▪ M. Miriani, M. Corredig, S. Iametti, F. Bonomi
Contribution title: "Soy proteins at oil-water interface: a fluorescence study"
Congress: Proteine 2010 **Date**: 8th-10th April 2010, Parma

- M. Miriani, M. Marengo, S. Barone, S. Iametti, F. Bonomi

Contribution title: "Elucidating the structural changes of bovine beta-lactoglobuline after adsorption on hydrophobic nanostructurated surface"
Congress:SIB-LLP 2010 **Date**: 28th May 2010, Varese

- M. Marengo, M. Miriani, F. Bonomi, S. Iametti, P. Ferranti, R. Hempelmann

Contribution title: "Magnetic nanoparticles for protein-protein interaction studies"
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- M. Miriani, M. Marengo, S. Barone, S. Iametti, F. Bonomi

Contribution title:"Structural and functional changes of bovine β-lactoglobulin - a food allergen - after adsorption on hydrophobic surfaces"
Congress:55th National Meeting SIB **Date**: 14th-17th September 2010, Milano

- M. Marengo, M. Miriani, S. Perego, A. Ferraretto, F. Bonomi, E. Prinz, R. Hempelmann, H. Frokayer

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- M. Miriani, M. Marengo, M. Corrado, S. Iametti, F. Bonomi

Contribution title: "Influence of the sorbent material and size upon structural and functional changes of bovine beta-lactoglobulin – a food allergen – after adsorption on hydrophobic surface"
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- M. Marengo, M. Matteo, F. Bonomi, S. Iametti, E. Prinz, R. Hempelmann, H. Frokayer

Contribution title: "Recognition and uptake of nanoparticle-conjugate food allergen by human monocytes"
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