GUSTAVO LEITE MILIÃO

A MULTI-SPECTROSCOPIC STUDY OF CHITOSAN-OVALBUMIN SUPRAMOLECULAR STRUCTURES FORMED AT DIFFERENT pHs

Dissertation submitted to the Universidade Federal de Viçosa, as partial fulfillment of the requirements of the Food Science and Technology Graduate Program for obtaining the degree of *Magister Scientiae*.

Advisor: Eduardo Basílio de Oliveira

Co-advisor: Jane Sélia dos Reis Coimbra

VIÇOSA – MINAS GERAIS

2019

T

Milião, Gustavo Leite, 1993-

M644m 2019

A multi-spectroscopic study of chitosan-ovalbumin supramolecular structures formed at different pHs / Gustavo Leite Milião. – Viçosa, MG, 2019.

80 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.

Inclui apêndices.

Orientador: Eduardo Basílio de Oliveira.

Dissertação (mestrado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. Análise espectral. 2. Interações. 3. Colóides. 4. Biomoléculas. 5. Proteínas. 6. Polissacarídeos. 7. Ovalbumina. 8. Quitosana. I. Universidade Federal de Viçosa. Departamento de Tecnologia de Alimentos. Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. II. Título.

CDD 22. ed. 664.07

GUSTAVO LEITE MILIÃO

A MULTI-SPECTROSCOPIC STUDY OF CHITOSAN-OVALBUMIN SUPRAMOLECULAR STRUCTURES FORMED AT DIFFERENT pHs

Dissertation submitted to the Universidade Federal de Viçosa, as partial fulfillment of the requirements of the Food Science and Technology Graduate Program for the degree of *Magister Scientiae*.

APPROVED: July 19th, 2019.

Gustavo Leite Milião
Gustavo Leite Milião
Author

Eduardo Basílio de Oliveira
Advisor

“NÃO DEVEMOS NOS ORGULHAR DE SERMOS MELHORES DO QUE OS OUTROS, E SIM MELHORES DO QUE JÁ FOMOS.”

JAMES C. HUNTER

ACKNOWLEDGEMENTS

Helen Keller once said that “Alone we can do so little; together we can do so much”, and the progression of this work would have been so much harder if I had not had the support of some people that I am more than greatful to. Then, I would like to thank:

God for giving me the strength to keep fighting for my goals.

My beloved family, especially my parents – Márcio Rodrigues Milião and Verônica de Fátima Leite Milião – and sister – Georgia Leite Milião, who always believed in me, even more than myself. Without their support and love I would not have made it through.

My relatives, who give all support that I need to keep on going. Especially my cousin Mariana, who I consider much more as a sister.

My dear advisor, professor Eduardo Basílio de Oliveira, who was responsible for my professional growth as a scientist. Thank you for believing that I was capable of working in the team. He has been more than an advisor during the last twenty four months, he showed himself to be a great friend. I could not be more thankful that I was one of your students!

Universidade Federal de Viçosa, Departamento de Tecnologia de Alimentos, and Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos for providing the training and opportunities that allowed me to become a better food scientist.

The financial support agencies – CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the scholarship along the past two years, CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais).

My co-advisor professor, Jane Sélia dos Reis Coimbra, who is always willing to provide outstanding advices to help her students to become better people.

The members of my advisory committee, Alvaro Vianna Novaes de Carvalho Teixeira and Éverton de Almeida Alves Barbosa, who certainly will provide valuable advices to improve the quality of this dissertation.

The professors Nilda de Fátima Ferreira Soares, Éber Antônio Alves Medeiros, and Gustavo Bressan for allowing our group to perform some experiments in the facilities coordinated by them.

The Centro Nacional de Pesquisa em Energia e Materias (CNPEM) for letting us to use some facilities in the Laboratório Nacional de Biociências (LNBio).

My co-workers from Laboratório de Estudo Materiais Alimentares (LEMA) e Laboratório de Operações e Pocessos (LOP) for all support and kind help throughout my research. Especially, I would like to thank: the little Ana Paula Hanke and Michele Harumi Omura for being so supportive and providing so many good moments to make this pathway 'softer'; Érica Cardoso, Layla Alves, Vitor Alledi da Rocha, Kely de Paula Correa, Monique Ellen Torres, Guilherme Mendes da Silva, José Antônio de Queiroz Lafetá Junior, and Davi Rocha Bernardes de Oliveira for the good moments during our funny lunch times; Talita Reis Teixeira e Silva for keeping our lab always organized; and last but not least, Danielle Ferreira and Thomás Valente de Oliveira for their support and precious friendship.

My dear co-workers and greatest friends, Douglas Fernando Balbino and Lucas de Souza Soares, who not only helped me with my research but also showed me the meaning of the word 'friendship' at the highest level.

My friends from the Men's volleyball team. They were extremely important throughout my time as a master student.

My friends, Arthur Amaral, Camila Gonçalves, Carlos Henrique Junqueira, Jean Carrara, Larissa Monteiro, Luiza Monteiro, Marciano Marques, Maria Júlia Pimenta, Nathália Medeiros, Thiago Quintão, Thiago Strutzel, and so many others for their patience, support and encouragement.

TO ALL OF YOU, I AM MORE THAN GREATFUL!

ABSTRACT

MILIÃO, Gustavo Leite, M.Sc., Universidade Federal de Viçosa, July, 2019. **A multi-spectroscopic study of chitosan-ovalbumin supramolecular structures formed at different pHs**. Advisor: Eduardo Basílio de Oliveira. Co-advisor: Jane Sélia dos Reis Coimbra.

Proteins and polysaccharides are important biomolecules used in food because of their techno-functional properties as thickening, gelling and, in the case of proteins, emulsifiers/foaming agents. However, proteins are unstable at extreme processing conditions (high temperatures, presence of organic solvents and proteolytic agents), which limits their application in food formulations. An alternative to overcome this problem is the use of supramolecular structures from protein-protein and protein-polysaccharides interactions. In this research, the latter type of association was investigated. Protein-polysaccharide associative interactions allow the formation of supramolecular structures, which, in turn, present even better biological and physicochemical features, making the study of such chemical species, especially in a molecular basis, relevant for a variety of applications. However, research about the association of the protein ovalbumin (OVA) and the polysaccharide chitosan (CHS), is still scarce, especially in the food science field. Then, OVA-CHS interaction in aqueous solution of chloride acid, at different pH values (4.0 and 6.0), was studied in order to evaluate if the polysaccharide presence would cause any expressive conformational changes in the overall protein structure. Therefore, a multi-spectroscopic approach, using UV-Vis and fluorescence to analyze the tertiary structure of OVA, conjointly with FT-IR and circular dichroism to study possible secondary structure modifications, was undertaken. UV-Vis and fluorescence results showed that the biomolecules interacted at some molecular level, forming complexes without a major modification in the microenvironment polarity of the protein. Furthermore, the fluorescence analysis suggested that the quenching mechanism of chitosan is likely static. Moreover, CD and FT-IR spectroscopies revealed that changes in the secondary structure of OVA upon interacting with CHS depended on the concentration of the polysaccharide. This study showed that the biomolecules could interact at both pH values forming complexes that could be applied in food, pharmaceutical, and medical applications. Such research allowed the comprehension of basic and advanced science, which contributed to the researchers' professional and (most important) human resource development.

Keywords: Protein. Polysaccharide. Interaction. Spectroscopies. Supramolecular structures.

RESUMO

MILIÃO, Gustavo Leite, M.Sc., Universidade Federal de Viçosa, julho de 2019. **Um estudo multi-espectroscópico de estruturas supramoleculares de quitosano-ovalbumina formadas em diferentes pHs**. Orientador: Eduardo Basílio de Oliveira. Coorientadora: Jane Sélia dos Reis Coimbra.

Proteínas e polissacarídeos são importantes biomoléculas usadas em alimentos devido às suas propriedades tecno-funcionais como agentes espessantes, gelificantes e, no caso de proteínas, emulsificantes/espumantes. No entanto, as proteínas são instáveis em condições extremas de processamento (altas temperaturas, presença de solventes orgânicos e agentes proteolíticos), o que limita sua aplicação em formulações de alimentos. Uma alternativa para superar este problema é o uso de estruturas supramoleculares formadas a partir de interações proteína-proteína e proteína-polissacarídeos. Nesta pesquisa, o último tipo de associação foi investigado. As interações associativas proteína-polissacarídeo permitem a formação de estruturas supramoleculares, as quais, por sua vez, apresentam características biológicas e físico-químicas ainda melhores, tornando o estudo dessas espécies químicas, a nível molecular, relevante para uma variedade de aplicações. Entretanto, pesquisas sobre a associação da proteína ovalbumina (OVA) e do polissacarídeo quitosano (CHS), ainda são escassas, principalmente no campo da ciência dos alimentos. Então, a interação OVA-CHS em solução aquosa de ácido clorídrico, em diferentes valores de pH (4,0 e 6,0), foi estudada para avaliar se a presença do polissacarídeo causaria alterações conformacionais expressivas nas estruturas da proteína. Assim, uma abordagem multi-espectroscópica, utilizando UV-Vis e fluorescência para analisar a estrutura terciária de OVA, conjuntamente com FT-IR e dicroísmo circular para estudar possíveis modificações da estrutura secundária, foi realizada. Os resultados de UV-Vis e fluorescência mostraram que as biomoléculas interagiram a nível molecular, formando complexos sem expressiva modificação na polaridade do microambiente da proteína. Além disso, a análise de fluorescência sugeriu que o mecanismo de supressão da OVA pelo CHS é provavelmente estático. Ainda, as espectroscopias de CD e FT-IR revelaram que mudanças na estrutura secundária da OVA ao interagir com CHS dependiam da concentração do polissacarídeo. Este estudo mostrou que as biomoléculas puderam interagir em ambos os valores de pH, formando complexos que poderiam ser aplicados em aplicações alimentares, farmacêuticas e médicas. Essa pesquisa permitiu a compreensão de ciência básica e avançada, o que contribuiu para o desenvolvimento profissional e (mais importante) dos recursos humanos do pesquisador.

Palavras-chave: Proteína. Polissacarídeo. Interação. Espectroscopias. Estruturas supramoleculares.

LIST OF FIGURES

CHAPTER ONE

Figure 1 – Molecular structures of cellulose (a) chitin (b) and chitosan (c).

Figure 2 – Primary sequence showing the secondary structure of ovalbumin.

Figure 3 – Tertiary structure ovalbumin.

Figure 4 – Maillard reaction pathways

Figure 5 – Michelson interferometer scheme

Figure 6 - Far-UV spectra pattern for α-helices, β-sheet and random coil.

CHAPTER TWO

Figure 1 – Absorption and emission spectra of OVA in the presence of different CHS concentrations at pH 4,0 (macromolecules are positively charged) and pH 6,0 (macromolecules are oppositely charged).

Figure 2 – Stern-Volmer plot of OVA with increasing concentrations of CHS.

Figure 3 – Ribbon structure (A) and Connolly surface structure of the OVA (B). Green and orange colors represent the tryptophan and tyrosine residues, respectively. These images were elaborated with data from the PDB entry 1OVA [33], without any modification of the crystal atomic coordinates, using (Pymol, version 2.2) software package.

LIST OF TABLES

CHAPTER ONE

Table 1 – Studies related to the potential applicability of chitosan and its derivatives in the medical-pharmaceutical industries.

Table 2 – Chitosan application for edible films formation in the food industry.

Table 3 – Studies related to the characterization and industrial applications of ovalbumin.

Table 4 – Studies on the formation and aplication of associative interactions via Maillard and complex coacervation between proteins and polysaccharides.

Table 5 – Studies related to the intermolecular interaction between OVA and CHS.

CHAPTER TWO

Table 1 - Percentages of different elements of the secondary structure of OVA in the absence and presence of CHS (1.0 - 3.0 $\mu mol \cdot L^{-1}$), at pH 4.0 and 6.0 (T = 25 °C).

SUMMARY

General introduction 12
Objectives 14
General objective 14
Specific objectives 14
References 16
Chapter one – State of the art 17
1. State of the art 18
1.1. Chitin and chitosan – molecular and physicochemical characteristics and applications 18
1.2. Ovalbumin 25
1.3. Supramolecular structures formed from protein polysaccharides interactions 31
1.3.1. Conjugates 32
1.3.2. Complex coacervates 33
1.4. Supramolecular structures formed between ovalbumin and chitosan 36
1.5. Spectroscopic techniques used to evaluate protein conformational changes upon interacting with ligands 40
1.5.1. UV-Vis spectroscopy 40
1.5.2. Fluorescence spectroscopy 41
1.5.3. Fourier transform infrared (FT-IR) spectroscopy 41
1.5.4. Circular dichroism (CD) spectroscopy 43
References 45
Chapter two - Manuscript 52
A multi-spectroscopic study of chitosan-ovalbumin supramolecular structures formed at different pHs 52
Abstract 54
Abbreviations and symbols 55
1. Introduction 56
2. Materials and methods 58
2.1. Materials 58
2.2. Preparation of ova and chs dispersions 58
2.3. Spectroscopic approaches 59
2.3.1. UV-Vis absorption spectroscopy 59

2.3.2. Fluorescence emission spectroscopy 59
2.3.3. Fourier transform – infrared (FT-IR) spectroscopy 60
2.3.4. Circular dichroism (CD) spectroscopy 60
3. Results 61
3.1. UV-Vis absorption spectroscopy 63
3.2. Intrinsic fluorescence spectroscopy 63
3.3. Fourier transform – infrared (FT-IR) spectroscopy 64
3.4. Circular dichroism (CD) spectroscopy 65
4. Discussion 66
5. Conclusion 72
Acknowledgements 72
References 73
General conclusion 77
Appendix 79

GENERAL INTRODUCTION

Polysaccharides and proteins are naturally occurring macromolecules in vegetable and animal raw materials, that are frequently used for industrial purposes, including food formulations. This is because such biopolymers, besides the nutritional role, possess techno-functional properties such as emulsifying, gelling, and foaming properties. Protein-polysaccharide associative interactions allow the formation of supramolecular structures, which, in turn, present even better biological and physicochemical features, making the study of such chemical species, especially in a molecular basis, relevant for a variety of applications. Upon researching at the Scopus database for "protein-polysaccharide" AND "interactions", over 500 papers have been published in order to understand the functionality of the formed supramolecular structures. In addition, a more refined search, including the word "food", showed that approximately 150 studies about protein and polysaccharides association have been reported only in the food field. Indeed, many papers have shown the phase behavior of complex structures originated from the interaction between different polysaccharides and proteins as well the potential applicability of such systems as either bioactive carriers or stabilizers agents in food formulated products. To mention only some emblematic cases, recent studies have reported the association of whey protein–pectin [1], soy protein isolate–gum acacia [2], soy protein hydrolysates–locust beam gum [3], lysozyme–carboxymethylcellulose [4], myofibrillar protein–kappa or lambda carrageenan [5], illustrating how widespread is the possibility of combination between proteins and polysaccharides.

Although the literature abounds of reports on the importance of supramolecular structures obtained from proteins and polysaccharides interaction, to the best of our knowledge, research about the association of two specific biomolecules, the protein ovalbumin (OVA) and the polysaccharide chitosan (CHS), is still scarce, especially in the food science field. Ovalbumin is the major egg-white protein [~55% (m/m)], which is used as a standard to study the allergenicity, properties, and structure of other proteins. Furthermore, OVA presents itself as a food ingredient with high thermal and surface features, which allows it to be used as an emulsifying, foaming, and/or gelling agent. Chitosan is a biocompatible, biodegradable, and non-toxic polysaccharide obtained from chitin deacetylation process. Amino groups ($-NH_2$) present on the CHS chains may become protonated ($-NH_3^+$) in acid aqueous media, conferring unique properties to this polysaccharide. Indeed, chitosan is the only existing cationic polysaccharide that, due to its versatile physicochemical and biological properties, has been widely used in the agriculture, cosmetic, papermaking, textile, tissue engineering, biomedical, and food sectors [6,7]. Although both biopolymers can be used individually in different

applications, e.g., as bioactives carriers and/or colloidal systems stabilizers, the study of association between OVA and CHS is of great interest to understand if new biomaterials with better physicochemical and biological properties could be formed. Therefore, in order to complement the few studies found [8,9], our research team sought to evaluate the interaction between these biopolymers at two pH values, in which both macromolecules would be positively charged (pH 4.0) or oppositely charged (pH 6.0).

Then, this present research was intended to understand the mechanisms of interaction and if possible conformational changes in the protein would occur upon OVA-CHS association. In order to achieve these goals, a multi-spectroscopic approach was undertaken, using UV-vis, Fourier transform infrared (FT-IR), circular dichroism (CD), and intrinsic fluorescence spectroscopies. Aside from the reports about ovalbumin and chitosan interaction, our study is the first one which seeks to understand the association of such biopolymers in a molecular level, with a set of distinct spectroscopic techniques, at two different pH values and, therefore, it hopefully will bring new information on what have been observed in literature so far.OBJECTIVES

General objective

To investigate the nature of intermolecular interactions between chitosan (CHS) and ovalbumin (OVA) dispersed in aqueous medium, at pH 4.0 and 6.0, as well as possible protein structural changes, through a multi-spectroscopic approach, comprising the UV-visible, Fourier transform infrared (FT-IR), circular dichroism (CD), and intrinsic fluorescence spectroscopies.

Specific objectives

- To evaluate possible protein secondary structure modifications, at a fixed concentration [5.0 $\mu mol \cdot L^{-1}$], in the presence of different chitosan concentrations [0.0, 1.0, 2.0, 3.0, and 4.0 $\mu mol \cdot L^{-1}$] – by circular dichroism and Fourier transform infrared spectroscopies, at pH 4.0 and 6.0, and at 25 °C;
- To qualitatively analyse possible protein tertiary structure and, consequently, microenvironment polarity modifications, at a fixed concentration [5.0 $\mu mol \cdot L^{-1}$], in the presence of different chitosan concentrations [0.0, 1.0, 2.0, 3.0, and 4.0 $\mu mol \cdot L^{-1}$] – by UV-vis spectroscopy, at pH 4.0 and 6.0, and at 25 °C;
- To qualitatively and quantitatively assess possible protein tertiary structure and, consequently, microenvironment polarity modifications, at a fixed concentration [20.0 $\mu mol \cdot L^{-1}$], in the presence of different chitosan concentrations [0.0, 3.0, 4.0, 5.0, and 6.0 $\mu mol \cdot L^{-1}$] – by intrinsic fluorescence spectroscopy, at pH 4.0 and 6.0, and at 25 °C;

- To correlate the spectroscopic results in order to understand the nature of OVA-CHS interaction in the supramolecular structures formation and if these intermolecular forces caused any modification in the protein structure, at pH 4.0 and 6.0.

References

[1] A. D. Setiowati, A. Rwigamba, P. Van Der Meeren, The influence of degree of methoxylation on the emulsifying and heat stabilizing activity of whey protein-pectin conjugates, Food Hydrocolloid. 96 (2019) 54-64.

[2] F. Xue, Y. Gu, Y. Wang, C. Li, B. Adhikari, Encapsulation of essential oil in emulsion based edible films prepared by soy protein isolate-gum acacia conjugates, Food Hydrocolloid. 96 (2019) 178-189.

[3] J. A. Lopes-da-Silva, S. R. Monteiro, Gelling and emulsifying properties of soy protein hydrolysates in the presence of a neutral polysaccharide, Food chem. 294 (2019) 216-223.

[4] Y. Pei, Z. Li, D. J. McClements, B. Li, Comparison of structural and physicochemical properties of lysozyme/carboxymethylcellulose complexes and microgels, Food Research International. 122 (2019) 273-282.

[5] A. M. Bakry, J. Huang, Y. Zhai, Q. Huang, Myofibrillar protein with κ-or λ-carrageenans as novel shell materials for microencapsulation of tuna oil through complex coacervation, Food Hydrocolloid. 96 (2019) 43-53.

[6] A. Muxika, A. Etxabide, J. Uranga, P. Guerrero, K. de La Caba, Chitosan as a bioactive polymer: processing, properties and applications, Int. J. Biol. Macromol. 105 (2017) 1358-1368.

[7] S. Islam, M. A. Rahman Bhuiyan, M. N. Islam, Chitin and chitosan: structure, properties and applications in biomedical engineering. Journal of Polymers and the Environment. 25 (2017) 854-866.

[8] W. Xiong, C. Ren, M. Tian, X. Yang, J. Li, B. Li, Emulsion stability and dilatational viscoelasticity of ovalbumin/chitosan complexes at the oil-in-water interface, Food Chem. 252 (2018) 181–188.

[9] W. Xiong, C. Ren, W. Jin, J. Tian, Y. Wang, B. R. Shah, J. Li, B. Li, Ovalbumin-chitosan complex coacervation: Phase behavior, thermodynamic and rheological properties, Food Hydrocolloid. 61 (2016) 895-902.

CHAPTER ONE

STATE OF THE ART

1. State of the art

1.1. Chitin and chitosan – molecular and physicochemical characteristics and applications.

Chitin [poly-β-(1→4)-2-acetamido-2-deoxy-D-glucopyranose] is a polysaccharide, primarily identified in 1884, which can be obtained from several living organisms, including insects, fungi, mollusk, and crustaceans [1]. However, shrimp and crab shells are the most important and available source of this biopolymer. In terms of quantity, chitin is the second most abundant polysaccharide found in nature, and approximately 100 bi tons of chitin are produced per year [2,3].

Regarding its applicability, chitin has low solubility in organic and aqueous solvents, which limits its use in industrial sectors. On the other hand, its transformation to chitosan [poly-β- (1-4) -2-amino-2-deoxy-D-glucopyranose] allows the diversification of the use of both biopolymers [1–3], which are natural, biocompatible, biodegradable and non-toxic. Chitosan may be obtained from the deacetylation step of chitin in alkaline medium or by the enzymatic action of chitin deacetylase. Although enzymatic deacetylation allows the product of interest to be obtained with greater purity, chemical deacetylation is preferred due to its low cost and higher production yield [2]. The chemical treatment is given by soaking the material in alkaline solution (40-50%) at 100-160 °C for a few hours. The processing time and the base concentration to be used will depend on the degree of acetylation (DA) and, consequently, the physicochemical characteristics of the desired chitosan compound. The DA is related to the percentage of units of N-acetyl groups present in the chitosan molecule. Generally, it is considered that when DA is less than 50%, chitin has been converted to chitosan [1], becoming soluble in acidic aqueous solutions and increasing its applicability. It is important to note that the deacetylation step is characterized not only by the removal of the N-acetyl groups but also by the hydrolysis of the chitosan chain, altering its average molecular mass and, therefore, its physicochemical properties.

Structurally, chitin and its derivative, chitosan (CHS), resemble cellulose (Figure 1), differing only in the substitution of the hydroxyl group (–OH) present on C2 of the cellulose by an acetamido ($–NHCOCH_3$) or amino ($–NH_2$) group, in chitin and chitosan, respectively.

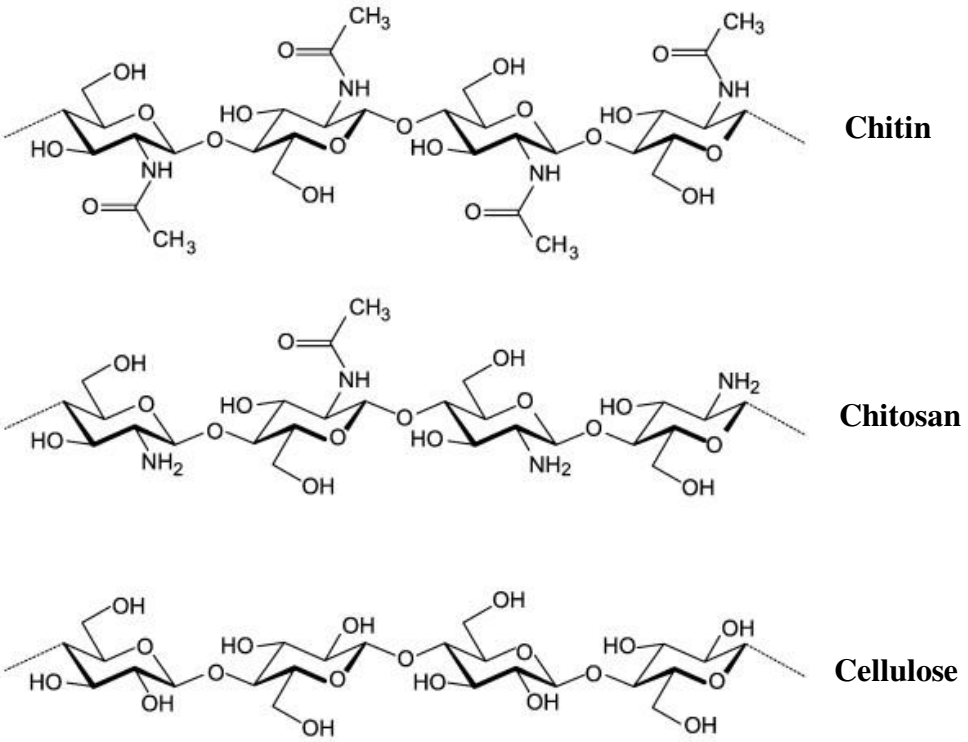


Figure 1 – Molecular structures of chitin, chitosan and cellulose [4].

This polysaccharide is the only one capable of acquiring a positive electrostatic charge, by means of protonation of amine groups, in acidic aqueous solution (Equation 1).

$$\mathbf{RNH_2 + H^+ \leftrightharpoons RNH^+_3} \qquad \text{pKa ~6.4} \qquad (1)$$

The presence of positively charged groups, together with the N-acetylated amine groups, gives chitosan an amphiphilic character, allowing the applicability increase of this biopolymer in several industrial applications.

Chitosan has been attracting researchers, academic, and industries attention for all these reasons, becoming a biopolymer with a wide biotechnological application in several sectors, such as biomedical engineering and bone tissue [5], fatty acid absorption [6] and the encapsulation, transport and controlled release of bioactive compounds for the food and pharmaceutical industries [1,7]. Table 1 presents studies on the potential use of chitosan in the medical-pharmaceutical industries.

Table 1 – Studies related to the potential applicability of chitosan and its derivatives in the medical-pharmaceutical industries.

Molecules	Objective	Results	Reference
Low molecular mass chitosan and soy proteins	To evaluate the effect of chitosan attachment on the reduction of the allergenicity of soy protein to develop further functional protein-polysaccharide conjugate.	• Enhanced conjugate antimicrobial activity • Good emulsifying properties of conjugates • The allergenicity of soy protein was greatly decreased by the attachment of chitosan through Maillard reaction.	[8]
Low molecular mass chitosan and tripolyphosphate	To encapsulate lutein to improve its water dispersability and also to increase its bio-availability.	• Increased lutein bioavailability • Increased postprandial lutein level in the plasma (54.5%), liver (53.9%) and eyes (62.8%) of mice fed on nanoencapsulated lutein.	[9]
Chitosan and curcumin	To prepare and characterize chitosan hollow nanospheres used for encapsulating anticancer drug curcumin.	• Average diameter: 500-1000 nm and inner cavity of 300-500 nm • Specific surface area: 101.91 $m^2 \cdot g^{-1}$ • Low cytotoxicity • Loading capacity: 63.9% • 87% of loaded curcumin was released over 50 h.	[10]

Chitosan oligosaccharides	To synthesize palladium nanoparticles coated with chitosan oligosaccharide and functionalize them with RGD peptide (arginine-glycine-aspartic acid) for effective accumulation in breast cancer cells.	• Increased particles biocompatibility • Increased nanoparticles accumulation in breast câncer cells, upon functionalizing • Improvement of photothermal therapeutic effects; • Good amplitude of photoacoustic signals → facilitates the imaging of tumor tissues → use of non-invasive photoacoustic tomography system.	[11]
Gelatin/carboxymethyl chitosan/nano-hydroxyapatite	To generate macroporosity in the scaffold using a combination of high-speed stirring induced foaming and freeze-drying method.	• Macroporous scaffold – better performances in terms of: o Pore size o porosity o water holding capacity o slow and sustained enzymatic degradation rate • Enhanced expression level of collagen type I, osteocalcin • Potential application in bone tissue engineering.	[12]

The research mentioned in Table 1 showed the applicability of chitosan mainly as a carrier agent of bioactive compounds (curcumin and lutein), a modulator of allergenic characteristics associated with soy protein, and a biocompatible material for use in animal tissue engineering along with the treatment of breast cancer. The studies highlighted the possibility of industrial application of chitosan and its derivatives. In addition to the aforementioned effects, chitosan has been studied for its action as a plasma lipid modulating agent. Rizzo et al. [13] described the effect of this polysaccharide on lipoproteins and on plasma lipids. The treatments were applied in 28 people with mean age of 63 ± 12 years who had triglyceride levels greater than 150 $mg \cdot mL^{-1}$. Patients received doses of 125 $mg \cdot day^{-1}$ of chitosan from fungal mycelia, during four months. Results showed that total cholesterol, LDL cholesterol, triglycerides were reduced by 8%, 2% and 19%, respectively. Furthermore, it was observed that HDL cholesterol levels increased by 14%. Due to its ability to become positively charged in acid media, chitosan, when present in the stomach (pH~2.0), can perform intermolecular interactions with cholesterol, fatty acid and triglyceride molecules present in foods. As a consequence of this physiological feature, there are many chitosan products on the market indicated for slimming treatments and as a reducing agent for blood cholesterol levels.

Chitosan applicability in food systems has grown over the years, but yet few studies have been published to show the use of chitosan as an ingredient and/or additive in formulations. Even though the application of chitosan as a food additive is not yet widespread, in 1991, the Codex Alimentarius allowed the use of this biopolymer as a clarifying and flocculating agent for juices and wines, thickening and stabilizing agent, fiber source and coating agent (FAO, 2003). In Brazil, the Agência Nacional de Vigilância Sanitária (ANVISA) is the organization responsible for regulating chitosan consumption, through Resolution n. 18 of April, 30th 1999, which presents claims of functional properties and/or product health. In this context, chitosan is defined as:

> "…dietary fiber which may be used with claims of reduction of fat and cholesterol, since the daily consumption of the product provides at least 3 g of chitosan (whether the food is solid) or 1.5 g (whether the food is fluid)".

In food industries, however, chitosan is mostly used for obtaining edible films that can act as protecting agents for food products. Table 2 brings some reports related to the application of chitosan in the food industry.

Table 2 – Chitosan application for edible films formation in the food industry.

Molecules	Objectives	Results	Reference
Chitosan, pea starch, tannic acid, thyme extract.	To study the antioxidant and physical properties of different polymeric matrices based on chitosan and pea starch, incorporating a thyme extract (TE) rich in polyphenols.	• Antioxidant films made up of chitosan or starch containing thyme extract were obtained. • Microstructural and physical properties of the films changed upon thyme extract addition • Polyphenols-chitosan interaction enhanced films mechanical response • Chitosan films preserved antioxidant activity of thyme extract.	[14]
Carboxymethyl chitosan (CMC), chitosan hydrochloride (CHC), zein and epigallocatechin gallate (EGCG).	To incorporate epigallocatechin in chitosan nanoparticles coated with zein for active packaging.	• EGCG incorporated in zein/chitosan nanoparticles presented higher DPPH scavenging activity than the control • Better mechanical properties • Potential use as photoxidation protecting agents.	[15]

Gelatin, chitosan, plant ethanolic extracts: guarana seeds, leaves of boldo-do-chile, cinnamon barks and leaves of rosemary.	To study the effect of incorporated plant ethanolic extracts into edible films prepared with blends of gelatin and chitosan	• Increased film elasticity • Biopolymers mixture reduced water vapor permeability • Good antioxidants properties and growth inhibition of *Escherichia coli* and *Staphylococcus aureus* • Potential use as active packaging.	[16]
Low molecular mass chitosan and glycerol (plasticizer).	To study the effects of chitosan-based coatings applied by dipping or spraying on the quality attributes of commercial ready-to-eat baby carrots stored at 4 °C under modified atmosphere packaging conditions	• Combination of modified atmosphere packaging and chitosan coating promoted better results • Microbial spoilage in carrots was delayed • Chitosan coatings exhibited positive effects on the colour and texture of carrots • Improvement of shelf-life.	[17]
High molecular mass chitosan and maqui *berry* (*Aristotelia chilensis*)	To evaluate chitosan edible films incorporated with maqui berry extracts for the inhibition growth of some bacterial strains and improvement of oxidative stability	• The films presented antibacterial properties against several spoilage bacteria • Improvement of antioxidante activity of chitosan films • Potential use as active films.	[18]

In general, the studies sought to evaluate chitosan application effects on the formation of films, in order to improve the physical properties of such materials (reduce water vapor permeability, improve elastic characteristics, etc.) and incorporate antioxidant antimicrobials compounds. All papers obtained positive results in terms of improvement of the physical properties and reduction of the deterioration time of the films, showing that the chitosan can be a good alternative to be used as an active packaging.

1.2. Ovalbumin

Ovalbumin (OVA) is a phosphorylated globular glycoprotein produced in chicken oviduct and present in a larger amount (about 55%, m/m) in egg white [19]. The complete sequencing of amino acid residues present in OVA was performed by Nisbet et al. [20]. The study revealed that the protein consists of 385 amino acid residues (Figure 2), showing a molecular mass of approximately 45 kDa and isoelectric pH of 4.5. Among egg white proteins, OVA is the only one that has just one disulfide bond – between Cys-73 and Cys-120 residues – along with four free sulfhydryl (-SH) groups within the protein [21,22]. When OVA is unfolded such sulfhydryl groups may be exposed, allowing the formation of new covalent bonds and/or intra/intermolecular interactions, altering their physicochemical and techno-functional properties (gelling, emulsifying and foaming, for example).

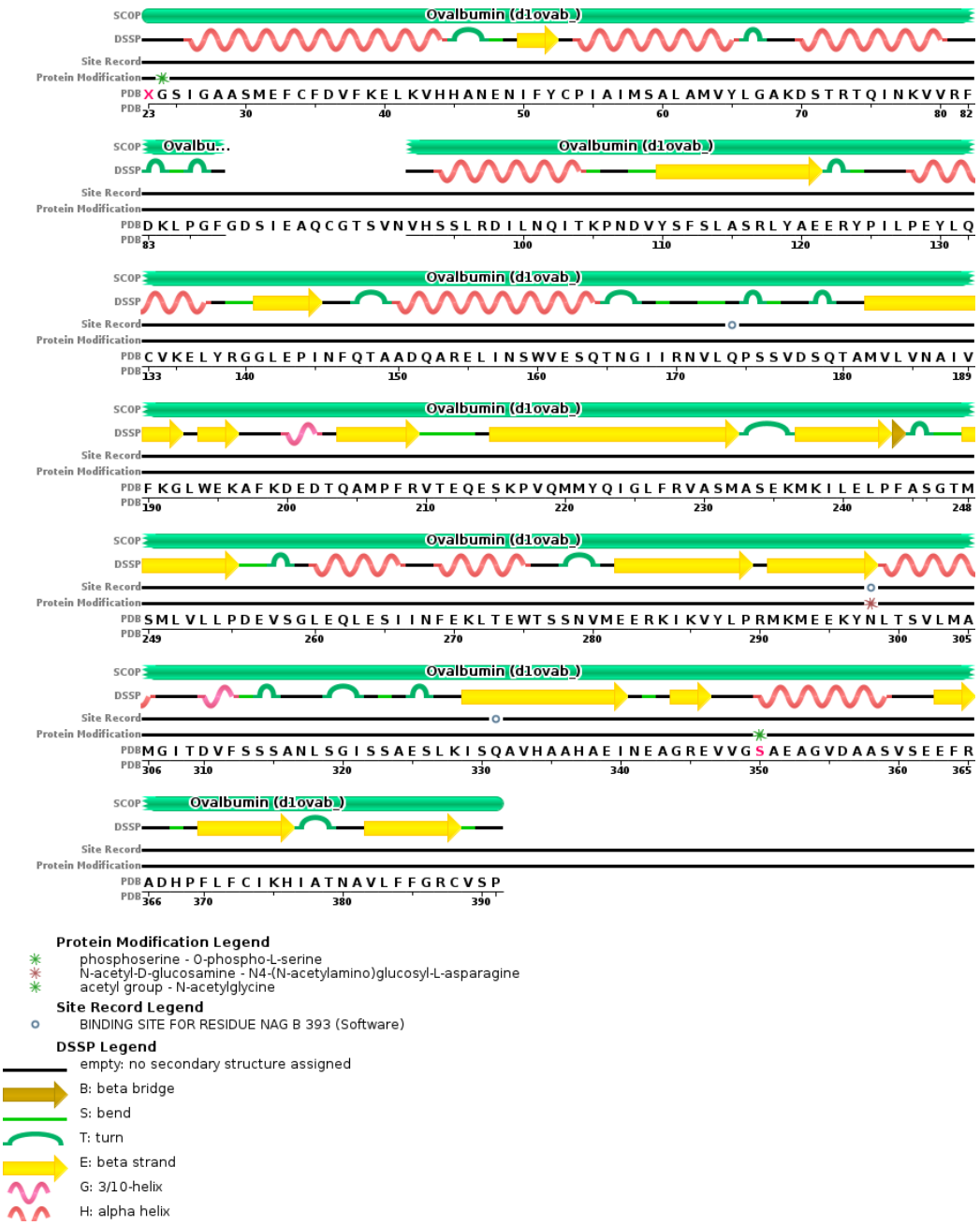


Figure 2 – Primary sequence showing the secondary structure of ovalbumin. Retrieved from: *Protein Data Bank (*PDB) - http://www.rcsb.org/3d-view/1OVA/1

In addition to the acetyl (-CH_3) group attached to the N-terminal end, there are three other post-translational modification sites in OVA. Part of the glycoprotein structure consists of carbohydrate molecules (two molecules of N-acetyl-glucosamine and four mannose units) attached to the amino acid residue Asn-292 [20]; and still, there are two phosphorylated serine residues in the protein. Figure 3 shows the tertiary structure of ovalbumin.

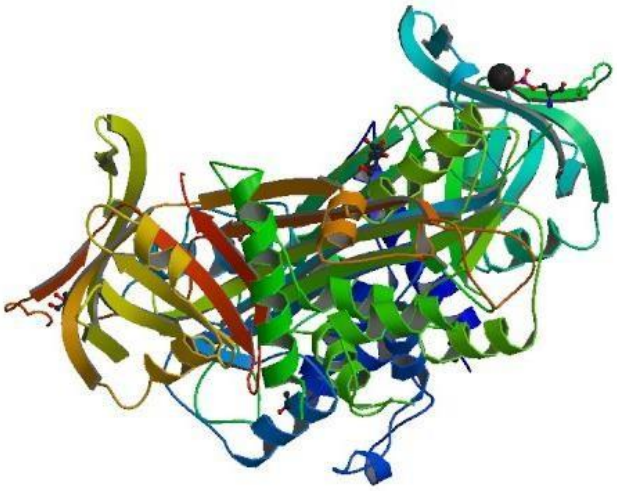


Figure 3 – Tertiary structure ovalbumin.
Retrieved from: *Protein Data Bank (*PDB) - http://www.rcsb.org/3d-view/1OVA/1

OVA is widely used as a standard in studies of protein structure and properties, and as an experimental model of research related to allergy, due to its ready availability in large quantities [23]. The interest in OVA applications by the food industry has been increasing due to its hydrophobicity and net charge, which allows its use as a surface active agent in colloidal systems [24]. Techno-functional properties presented by OVA stimulate researchers to evaluate the possibilities of its industrial application as emulsifying, stabilizing and gelling agents in formulations. Moreover, studies have proposed to produce supramolecular structures of OVA with other proteins or polysaccharides, in order to evaluate if the new formed biomaterials present better physicochemical and/or biological properties when compared to the biomolecule alone. In this context, Table 3 presents studies related to the OVA characterization and application in food systems.

Table 3 – Studies related to the characterization and industrial applications of ovalbumin

Biomacromolecules	Objectives	Main results	Reference
Ovalbumin	To describe the sequence determination of the complete ovalbumin molecule.	• Ovalbumin has four sites of postsynthetic modification • The heptapeptide released during the conversion of ovalbumin to plakalbumin corresponds to residues 346-352 • OVA polymorphism that has been shown to involve an Asn →Asp replacement is due to a mutation at Asn-311.	[20]
	To study the crystal structure of uncleaved ovalbumin at 1.95 Å.	• The final refined model presented: o 4 OVA molecules o 678 water molecules and a single metal ion o Crystallographic R-factor of 17.4% for all reflections between 6.0 e 1.95 Å • The peptide that is homologous to the reactive centre of inhibitory serpins adopts an a-helical conformation.	[25]
	To evaluate the effect of phosphorilation on the interfacial properties of OVA in the O/W emulsions formation.	• The pI of phosphorylated OVA at low pH suggested its stability over a wider pH range • The equilibrium interfacial tension decreased from 17.359 $mN \cdot m^{-1}$ (OVA native) to 15.969 $mN \cdot m^{-1}$ (OVA phosphorilated) • Narrower size distribution with smaller particle size in phosphorilated ovalbumin stabilized emulsions	[26]

	To evaluate the effect of interaction of tannic acid in the emulsifying properties of ovalbumin.	• OVA surface activity decreased • Tannic acid decreased the emulsifying ability of ovalbumin • Emulsions stability increased in the presence of tannic acid.	[27]
	To evaluate structure change of OVA through fluorescence, circular dichroism and Fourier-Transform (FT) Raman spectroscopies during the storage period as well as its emulsifying and foaming properties.	• Percent of α-helix and β-sheet of OVA decreased • Emulsifying & foaming properties of OVA all decreased during the storage • Emulsifying & foaming properties of OVA all decreased during the storage.	[19]
	To investigate the interaction between ovalbumin and glutenins or gliadins in mixing and fermentation stages of Chinese steamed bread processing, and to check whether ovalbumin may improve final quality of CSB, such as specific volume and hardness.	• Ovalbumin has non-covalent interaction with glutenin • G' and G" of dough decreased • OVA was unfolded at high temperature, and cross-linked with gluten protein • Ovalbumin improved specific volume of Chinese steamed bread.	[28]
Ovalbumin and lysozyme	To study the influence of pH, NaCl concentration, and total protein mass ratio on the formation of coacervate complexes.	• Heteroprotein complex coacervates formed at pH 7.5 and razão mass ratio 1:1 • NaCl disfavored complexes formation and suppressed it at 300 mM • Average size: ~2 μm (globular structure) • Interaction: exothermic and spontaneous.	[29]

Ovalbumin, lysozyme, and carrageenan	To elucidate the influence of protein, pH, ionic strength and the ratio of protein/polysaccharide in the formation of complexes between egg white proteins (ovalbumin and lysozyme) and carrageenan.	• The increase in mass ratio (1:1 to 10:1) increased the pH values of complex formation • Decrease of G' values at ratios of 5:1 and 10:1 • Salt addition suppressed the electrostatic interactions • Potential application as food	[30]

The aforementioned studies deal with the characterization of ovalbumin in terms of aminoacid sequence, secondary and tertiary structure, crystallization, and post-translational modifications. Also, studies on the application of OVA, individually or in combination with other molecules, in foods are presented. Sang et al. [28] evaluated the functionality of ovalbumin during the processing of Chinese bread in order to investigate whether this protein interacts with wheat proteins (gliadins and glutenins) in the mixing and fermentation steps. Furthermore, it was evaluated if OVA was capable of improving the final quality of the bread in terms of specific volume and hardness, for instance. Rheological studies showed that ovalbumin reduced the storage (G') and loss (G") moduli of dough. Also, the maximum mass height has been improved along with the specific bread volume. Such conjoint analyses suggested that ovalbumin could be used to increase the loaf volume, improving the final quality of baked products.

Souza et al. [30] evaluated the effect of pH, ionic strength, and mass ratio between biopolymers (lysozyme:carrageenan and ovalbumin: carrageenan) in the formation of complex coacervates with potential applicability in foods. The supramolecular structures were characterized in terms of turbidity, thermodynamic parameters, and rheological properties. Results showed that with the augment in biopolymers mass ratio from 1:1 to 10:1, the transition (pH_c, $pH_{\varphi 1}$ and $pH_{\varphi 2}$) and formation points of complexes increased for the OVA/carrageenan system. In addition, the change in the ionic strength of the medium suppressed the formation of the structures due to the shielding effect caused by ions. The yield of complexes formed between ovalbumin and carrageenan was 79.6 ± 0.6%, for 3:1 mass ratio and 0.01 $mol \cdot L^{-1}$ NaCl. In this same proportion, the complexes presented maximum shear modulus, suggesting that complexes may act to modulate sensory attributes such as texture, consistency, and taste of food. Research proposing the study of protein-protein and protein-polysaccharides interactions to produce supramolecular structures has grown considerably in the academic and industrial areas due to their potential applicability in several sectors, highlighting, for example, their ability to carry and release bioactive compounds.

1.3. Supramolecular structures formed from protein polysaccharides interactions

Proteins and polysaccharides are important biomolecules used in food because of their techno-functional properties as thickening, gelling and, in the case of proteins, emulsifiers/foaming agents. However, protein instability through extreme processing conditions (high temperatures, presence of organic solvents and proteolytic agents, turbulent flow, etc.) is a limitation for the application of such macromolecule in food formulations [31].

The associative interaction between these biopolymers has been studied in order to find ways to overcome this instability and, consequently, to improve physical stability and texture features of foods. Proteins and polysaccharides may interact through two main types of associations: a) non-covalent attractive forces, which lead to the obtention of complexes/coacervates from physical interactions (electrostatic, H-bonding, hydrophobic, van der Waals) between the biomacromolecules; and b) covalent bonds, which allow the formation of conjugates from Maillard reaction [31,32].

1.3.1. Conjugates

Protein modifications with the purpose of improving the techno-functionality of these biomolecules can be obtained through chemical, enzymatic or physical treatments [31]. However, the use of chemical reagents is a limitation when choosing chemical treatments, since consumers are resistant to the application of additives that undergo chemical transformations in food formulations. Thus, obtaining conjugates from the Maillard reaction (Figure 4) becomes an alternative for altering the techno-functional properties of proteins. Supramolecular structures obtained from the glycosylation of proteins with polysaccharides can be used as carrier agents of bioactive compounds, emulsifying/foaming agents and stabilizers in the food industry. Many studies (Table 4) have reported about the formation of conjugates in order to improve physicochemical and techno-functional properties of proteins.

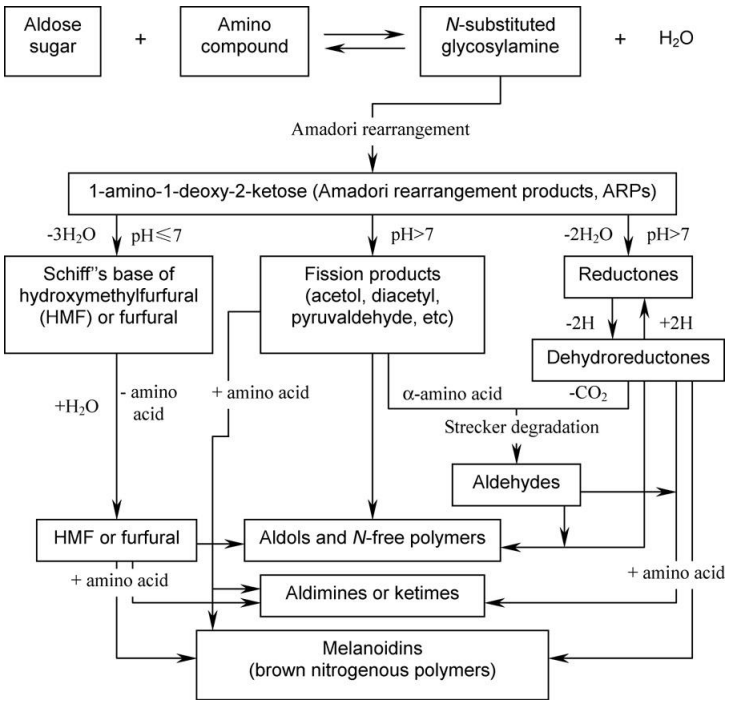


Figure 4 - Maillard reaction pathways adapted from Martins et al. [33].

1.3.2. Complex coacervates

Another way to obtain associative interactions between proteins and polysaccharides is through complex coacervation (CC). In this system, when two or more oppositely charged biopolymers interact (mostly through electrostatic interactions), phase separation is observed, being one phase denser and rich in biopolymers, and the other diluted and rich in solvent (mainly water). On the other hand, there may be precipitation of supramolecular structures in the aqueous medium which characterizes the formation of liquid-solid type phases. Even though the principal driving force of complex coacervation is the electrostatic association between biopolymers, a set of combined weak attractive interactions such as H-bonding, hydrophobic, and van der Waals may play important roles in the formation of such systems. Therefore, the interaction force between the biomacromolecules is the determining factor of the type of phase separation that will occur. In both cases, the structure of the proteins is kept intact which enables the use them in a range of applications in the pharmaceutical, cosmetic, and food sectors, mainly as bioactives carriers and colloid stabilizers [34]. Critical parameters (protein isoelectric point, temperature, mixing ratio, biomacromolecules density charge, chain flexibility and polyeletrolyte charge sequence distribution) must be taken into account, in order to obtain complexes coacervates [32,35,36]. Table 4 presents some studies involving the formation of conjugates and complexes and their application in the food and pharmaceutical industries.

Table 5 – Studies on the formation and aplication of associative interactions via Maillard and complex coacervation between proteins and polysaccharides.

Macromolecule A	Macromolecule B	Application	Reference
CONJUGATES FORMATION VIA COVALENT BONDING –MAILLARD REACTION			
ε-polylysine	Chitosan	Improvement of conjugate antimicrobial activity.	[37]
Whey protein isolate	Glucose	Improvement of conjugate termal stability and antioxidant properties.	[38]
Ovalbumin	Dextran	Improvement of curcumin bioavailability.	[39]
Sodium caseinate	Lactose	Enhancement of protein-polysaccharides emulsions stability to improve encapsulation efficiency of oil.	[40]
Canola protein isolate	Gum Arabic	Improvement of emulsifying properties of canola protein isolate.	[41]
Casein peptides	Gum Acacia	Improvement of emulsifying properties for oil-in-water emulsions.	[42]
Sodium caseinate	Corn fiber gum hydrolysate	Improvement of conjugate interfacial properties.	[43]
Casein	Dextran	Improvement of casein micelles stability and controlled release of lutein.	[44]
BSA	Beet pectin	Improvement of conjugates emulsifying properties.	[45]
Sodium caseinate	Corn starch hydrolysates	Stabilizer agent of emulsions containing resveratrol.	[46]
COMPLEX COACERVATES FORMATION VIA NON-COVALENT INTERACTIONS			
Casein	Pectin	Encapsulation and controlled release of acetaminophen.	[47]
Chitosan	Alginate	Encapsulation and controlled release of riboflavin (vit. B2).	[48]
Sodium caseinate	Gum Arabic	Encapsulation of EPA /DHA and utilization of the nanoparticles to enrich juice.	[49]
β-lactoglobulin	Lysozyme	Encapsulation of vitamin D3.	[50]

Gelatin	Cashew gum	Encapsulation of *echium oil*.	[51]
β-lactoglobulin	Lactoferrin	Encapsulation of vitamin B9.	[52]
Casein	Tragacanth gum	Encapsulation of β-caroten.	[53]
Soy protein isolate	Chitosan	Microencapsulation of polyunsaturated fatty acids from algae oil.	[54]
Gelatin	Gum Arabic	Encapsulation of shrimp lipid extract as a potential food coloring in yogurt.	[55]
Whey protein isolate	Chitosan	Encapsulation of garlic extract.	[56]

In Table 4, studies reported that the conjugates obtained through the Maillard reaction can be used for the purpose of improving proteins emulsifying properties, carrying bioactive molecules, and improving the antioxidant and antibacterial activity of proteins and polysaccharides. Moreover, most studies on complex coacervates are directly linked to the use of such systems in the encapsulation and controlled release of bioactive molecules.

1.4. Supramolecular structures formed between ovalbumin and chitosan

Ovalbumin and chitosan are biological macromolecules which can be used individually to help reducing the instability of colloidal systems, due to their techno-functional properties as emulsifying, gelling, foaming (in the case of OVA), and stabilizing agents. However, studies on the intermolecular interaction between these biomacromolecules have been developed to evaluate their effects in industrial applications such as in emulsion formulations and drug delivery. To the best of our knowledge, only three papers (Table 5) have described the association of OVA and CHS. A brief overview of what has been discovered on such studies is described in the following paragraphs.

Table 5 – Studies related to the intermolecular interaction between OVA and CHS.

Biomolecules	Objective	Caracterization	Main results	Reference
Ovalbumin and Chitosan	To prepare nanogels from biomolecules interaction upon heating, at pH 5.4 and mass ratio of 0.2.	• ζ potencial • UV-Vis absorbance (turbidity) • Dynamic Light Scattering • Transmission Electron Microscopy • Extrinsic fluorescence spectrometry (pyrene)	• OVA and CHS interacted forming nanogels (T = 80 °C/20 min) • Part of chitosan chains were trapped in the core (electrostatic interaction) and the rest was outside forming the shell • Nanospheres average size: 120 nm • Size distribution mantained unvariable for 90 days • Nanogels did not dissociate in pH range of 2-10.5 • Dispersibility, size, and hydrophobicity/hydrophilicity of the nanogels are pH-dependent.	[57]
	To evaluate the influence of pH, ionic strength, and ovalbumin/chitosan ratio on the phase behavior, thermodynamic and rheological properties of the complex coacervates.	• Turbidity • ζ potential • Isothermal Titration Calorimetry • Confocal laser scanning microscope (CLSM) • Rheological measurements.	• Complex coacervates formation – eletrostatic interaction • pH range ~ 4,61 - 6,40 • Ionic strength: ○ ≤ 200 mM NaCl → coacervates ○ > 200 mM NaCl → shielding effect • increase of OVA/CS ratio from 1:1 to 10:1 caused the decrease of critical pH (pH_c and pH_φ) • OVA/CS interaction: spontaneous and exothermic, at pH 4.0 and pH 5.5.	[58]

To explore the effect of OVA-CS interaction on oil in water (O/W) emulsions stability, at pH 4.0 and 5.5, and evaluate the interfacial dilatational properties of OVA/CS absorbed layers at O/W interface through rheological measurements.	• Particle size measurement of emulsions • Optical microscopy • Creaming index (CI%) • Rheological measurement of emulsions • Dynamic interfacial surface pressure (π) and interface viscoelasticity measurement.	• Improvement of emulsions stability during 14 days, at 25 °C • Complex formation: increased emulsions G' and η_{app} (pH 5.5) → reduced CI% • Increased dilatational elastic modulus (*Ed*): reduced the possibility of coalescence.	[24]

Xiong et al. [58] observed that the complexes formed at pH 4.0 presented $\Delta H = -(8.802 \pm 2.212) \cdot 10^4$ kcal·mol^{-1} and $T \cdot \Delta S = -8.642 \cdot 10^4$ kcal·mol^{-1}. On the other hand, at pH 5.5, $\Delta H = -(1.025 \pm 0.0337)$ kcal·mol^{-1} and $T \cdot \Delta S = 4.261$ kcal·mol^{-1}, suggesting that electrostatic association would be the main driving force on OVA-CHS complex coacervation. Furthermore, ≤ 200 mmol·L^{-1} NaCl favored the structures formation, whereas shielding effect in the electrically charged polymers was observed at ≥ 200 mmol·L^{-1} NaCl, which may have reduced coacervate complexes formation.

Xiong et al. [24], based on the aforementioned research, evaluated the effect of complexes formation on the stability of emulsions, highlighting the mechanisms involved in stabilization, at pH 4.0 and 5.5. Dispersions of ovalbumin and chitosan (1% m/v) were prepared and mixed in a ratio of 3:1 (m/m), under agitation for 30 min. The emulsions were prepared with soybean oil ($\emptyset_D = 0.2$), being the continuous phase composed of OVA or OVA/CHS mixture, in a total biopolymers concentration of 0.3% (m/m). The study provided an understanding of the stability of the emulsions prepared in the presence of the complexes (pH 5.5). In this condition, the viscoelastic properties of the emulsions were improved, reducing droplets mobility and preventing destabilization mechanisms, such as creaming and coalescence. Moreover, the presence of chitosan reduced the rates of diffusion, adsorption/permeation and OVA rearrangement at the interface. Furthermore, the complexes formed adsorption layers thicker than OVA alone, which made it possible to obtain a larger dilational modulus (providing greater mechanical strength) due to the intermolecular interactions between the biopolymers. Therefore, the formation of complexes from CHS and OVA showed good performance in relation to the stability of the emulsions, allowing their use as stabilizing agents in colloidal systems.

Yu et al. [57] also evaluated the influence of pH on the stability of nanogels formed from the intermolecular interaction between OVA and CHS. In order to prepare the nanogels, an acid dispersion of chitosan (0.75% w/v) was titrated in a dispersion of ovalbumin (0.02% w/v) under constant stirring until the CHS / OVA mass ratio reached 0.2. The mixture pH was then adjusted to 5.4, in order to ensure that the protein was above its isoelectric point (pI ~ 4.5), with predominantly negative charges, and chitosan below its pKa (~6.4) with predominantly positive charge, in order to promote nanogels formation. Results indicated that part of the chitosan chain was trapped inside the nanogels, while the rest of the chain remained outside to cover the structures. The size distribution of the nanogels was not expressively altered after a storage period of 90 days at 4 °C and there was also no structures dissociation in the pH range of 2 to 10.5. Also, even in the absence of antiseptic, the nanogels did not present microbiological

deterioration, suggesting that chitosan maintained its bacteriostatic activity. The pH dependence of phycochemical properties such as the dispersibility, size and hydrophobicity/hydrophilicity of nanogels allow them to be used as carrier agents in the pharmaceutical and medical industries.

Aside from what has been described, our research team aims at understanding the interaction between OVA and CHS at a molecular level. Therefore, we propose to perform a multi-spectroscopic analysis of the structures formed after ovalbumin-chitosan association, at two different pH values (4.0 and 6.0). Nowadays, none of the reports in literature involving these two biopolymers has proceeded with such experiments, and we believe that understanding the interaction of OVA and CHS and the protein possible conformational changes upon complexation could increase the applicability of these supramolecular structures in the pharmaceutical, cosmetics, medical, and food industries.

1.5. Spectroscopic techniques used to evaluate protein conformational changes upon interacting with ligands

Protein functions are directly related to their tridimensional structure. Therefore, slight conformational changes of such macromolecules can affect proteins' functionality and; therefore, the quality of the product of interest. In order to evaluate structural modifications in proteins, several sophisticated analytical methods have been developed with the aim to replace time consuming experiments such as protein solubility assay, free sulfhydryl content, and surface hydrophobicity. Spectroscopic techniques like UV-Vis, fluorescence, Fourier transform infrared, and circular dichroism have been widely studied as powerful tools to assess modifications in protein structures

1.5.1. UV-Vis spectroscopy

UV-Vis spectroscopy is used to qualitatively evaluate tertiary conformational changes in proteins. The most important chromophore groups, i.e., the chemical groups responsible for absorbing the UV-Vis radiation, are the aromatic aminoacids tryptophan (Trp), tyrosine (Tyr), and phenilalanine (Phe), which are highly sensitive to the environment where they are inserted. The spectral peaks of each aromatic aminoacid occur as follow: i) at 250-265 nm spectra are mostly due to Phe residues absorption; at 265-280 nm the peak formation is attributed to Tyr-Trp contributions, while at wavelengths greater than 285 nm the contributions are mainly due to Trp residues. Regarding the interaction between proteins and ligands, the appearance of peaks originates mostly from the $\pi \rightarrow \pi^*$ transition of the aromatic residues. Slight perturbations around these aminoacids residues could suggest that some sort of interaction would be

happening between a protein and a ligand, yielding possible changes in the structure of the protein. Therefore, UV-Vis spectroscopy is commonly used to assess complex formation and structural modifications on proteins [59].

1.5.2. Fluorescence spectroscopy

Fluorescence is one of the two phenomena which comprise molecular luminescence. Luminescence, in turn, is the emission of light from any substance that have been electronically excitated by radiation [60]. Regarding fluorescence spectroscopy, the absorption of electromagnetic radiation in the UV-Vis region by aromatic molecules promotes the emission of photons, which are electric fields traveling through space as tiny particles that contain energy. The transition between absorption and emission, and consequently fluorescence generation, can be well explained by the Jablonski diagram. Fluorescence spectroscopy has been widely used to study modifications in the tertiary structures of proteins. Similar to the UV-Vis spectroscopy, the aminoacids tryptophan, tyrosine and phenylalanine are the intrinsic chromophores responsible for absorbing the radiation, at the UV region, and emitting the photons at higher wavelengths (> 330 nm). More specifically, the intrinsic fluorescence of Trp is commonly used to assess the location of such aminoacid in a protein. This occurs because the indole group of Trp residues is highly sensitive to the solvent surrounding it. Generally, the emission from a residue within the protein's hydrophobic pocket happens at lower wavelengths when compared to residues located on the surface of the protein [59,60]. As it can be seen, fluorescence spectroscopy appears as an outstanding technique to evaluate protein unfolding and dynamics. Besides, it can also be applied to analyse the interaction of polypeptides and different ligands.

1.5.3. Fourier Transform Infrared (FT-IR) spectroscopy

Infrared spectroscopy corresponds to a technique which measures the rotational and vibrational motions of molecules due to specific energy levels at different frequencies. This type of spectroscopy is based on the absorbtion of energy by chemical bonds, especially stretching and bending motions. The infrared energy is related to the electromagnetic spectrum situated in the wavenumber range of 14290-200 cm^{-1}. However, for proteins structural characterizarion, the so called mid-infrared (4000-400 cm^{-1}) region is mostly used. As stated before, vibrational spectra arises from the energy absorption and consequently the transition between vibrational and rotational states of molecules. Such transition affects the molecules dipole moment, allowing the observation of spectra [59,61].

In proteins, the three most prominent bands for structural characterization are: i) amide I near 1,650 cm^{-1} (C=O stretch conjointly with weak C–N stretch and N–H bend), ii) amide II near 1,550 cm^{-1} (N–H bend and C–N stretch), and iii) amide III near 1,300 cm^{-1} (C–N stretch,

N–H bend, along with deformation vibrations of C–H and N–H) [29,30]. Since the many existing secondary structure in proteins are mostly stabilized by hydrogen bonding between the C=O and N–H of the aminoacids (which compose the backbone of the polypeptide) the amide I band is the most sensitive IR spectral region used to foresee proteins secondary structure. The equipment used to analyse samples by infrared spectroscopy is called infrared spectrometer. Basically, the central part of a FT-IR spectrometer is the Michelson interferometer (Figure 5).

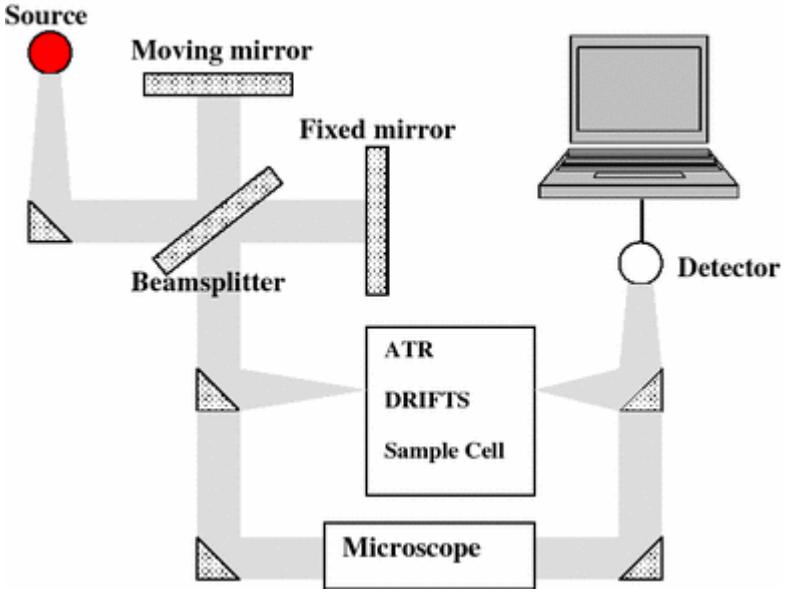


Figure 5 – Michelson interferometer scheme [61].

Briefly, the interferometer consists of two plane mirrors placed perpendicularly to each other, being one of them fixed and the other movable. In addition, a semi-transparent mirror (beamsplitter) is aligned with the light source. The beamsplitter is composed of a material that is capable of splitting the light in two halves, which in turn travel through different pathlengths. The two beams are reflected by the mirrors and, consequently, a time delay in one beam causes a phase difference when these beams are combined together in the detector site, creating the interferogram. The Fourier transform of the intensity spectrum of the source is, then, a interferogram measured as a function of time.

In terms of structural analysis, FT-IR spectra is widely used to evaluate conformational changes, especially secondary structures, of proteins when submitted to extreme process conditions (high temperature, acidic pH, shear stress, etc.) or when in contact with ligands. The analyses may be performed either in solid state or in aqueous solution. However, when FTIR absorption of proteins is measured in solutions, it can suffer a strong interference of water molecules absorption at 1640 cm^{-1} (amide I band). Therefore, in order to eliminate such effect,

the IR absorption of proteins is commonly undertaken in potassium bromide (KBr) pellets [59,61,62].

1.5.4. Circular dichroism (CD) spectroscopy

CD spectroscopy is a valuable technique used to evaluate the structure of macromolecules in solution. More specifically, circular dichroism presents a broad application in the characterization of proteins, especially for the secondary structures estimation. The equipment used to perform CD analysis is known as spectropolarimeters, and they measure the difference in absorbance between the left handed and right handed circularly polarized components. CD spectra rise primarily from chiral chromofores, i.e., molecules that are optically active. Since almost all aminoacids are chiral (except glycine), proteins may be easily characterized by circular dichroism.

In addition, this spectroscopic technique allows the assessment of proteins conformational changes, upon the interaction with ligands or when submitted to different extrinsic medium conditions. Theoretically, at wavelengths below 260 nm (far UV), the absorption of light is mostly due to the peptide bond, in which there is a wide n → π* transition (at ~ 220 nm) and an even more intense π → π* transition near to 190 nm. On the other hand, at wavelengths above 260 nm (near UV) the spectra arise mostly because of the aromatic aminoacids [63]. Therefore, depending on the region of study, modifications in both secondary and tertiary structures may be evaluated. Figure 6 presents the typical CD spectra pattern found for secondary elements (α-helices, β-sheet and random coil) of proteins.

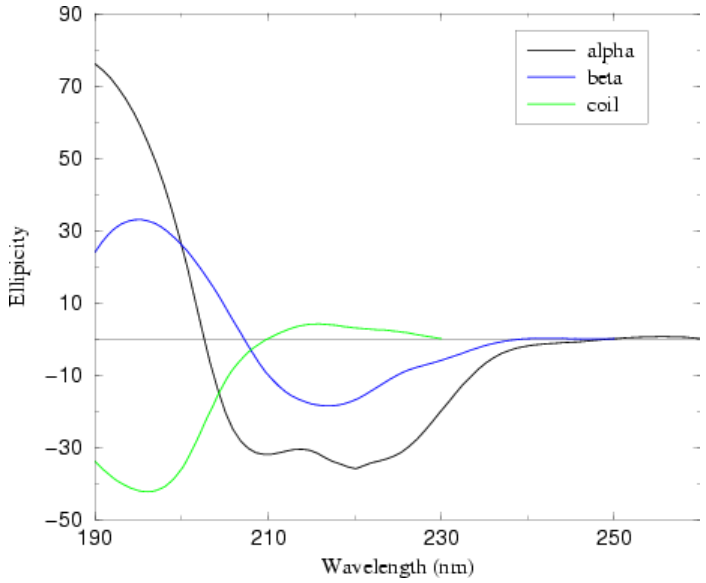


Figura 6 - Far-UV spectra pattern for α-helices, β-sheet and random coil.
Retrieved from: http://www.isbg.fr/biophysics-characterisation/circular-dichroism/?lang=fr

Commonly, positive peaks at 191-193 nm and doubled negative peaks at 208 and 222 nm are typical of proteins rich in α-helix regions. On the other hand, β-sheet structures present an intense positive band around 195-200 nm and a negative band at 216-218 nm. Furthermore, negative peaks at approximately 195-200 nm and a weaker band (positive or negative) between 215-230 nm are commonly found in proteins rich in random coils [59,63]. Noticeably, the application of CD spectroscopy can give rise to good insights about the folding status of proteins in solutions and identify changes in protein's structure upon the interaction of such macromolecules with ligands.

References

[1] I. Younes, M. Rinaudo, Chitin and chitosan preparation from marine sources. Structure, properties and applications, Mar. Drugs. 13 (2015) 1133–1174. doi:10.3390/md13031133.

[2] A. Muxika, A. Etxabide, J. Uranga, P. Guerrero, K. de la Caba, Chitosan as a bioactive polymer: Processing, properties and applications, Int. J. Biol. Macromol. 105 (2017) 1358–1368. doi:10.1016/j.ijbiomac.2017.07.087.

[3] V. Zargar, M. Asghari, A. Dashti, A Review on Chitin and Chitosan Polymers: Structure, Chemistry, Solubility, Derivatives, and Applications, ChemBioEng Rev. 2 (2015) 204–226. doi:10.1002/cben.201400025.

[4] L. Chiappisi, M. Gradzielski, Co-assembly in chitosan-surfactant mixtures: Thermodynamics, structures, interfacial properties and applications, Adv. Colloid Interface Sci. 220 (2015) 92–107. doi:10.1016/j.cis.2015.03.003.

[5] S. Islam, M.A.R. Bhuiyan, M.N. Islam, Chitin and Chitosan: Structure, Properties and Applications in Biomedical Engineering, J. Polym. Environ. 25 (2017) 854–866. doi:10.1007/s10924-016-0865-5.

[6] N. Panith, J. Wichaphon, S. Lertsiri, N. Niamsiri, Effect of physical and physicochemical characteristics of chitosan on fat-binding capacities under in vitro gastrointestinal conditions, LWT - Food Sci. Technol. 71 (2016) 25–32. doi:10.1016/j.lwt.2016.03.013.

[7] M. Fathi, Á. Martín, D.J. McClements, Nanoencapsulation of food ingredients using carbohydrate based delivery systems, Trends Food Sci. Technol. 39 (2014) 18–39. doi:10.1016/j.tifs.2014.06.007.

[8] M. Usui, H. Tamura, K. Nakamura, T. Ogawa, M. Muroshita, H. Azakami, S. Kanuma, A. Kato, Enhanced bactericidal action and masking of allergen structure of soy protein by attachment of chitosan through Maillard-type protein-polysaccharide conjugation, Nahrung - Food. 48 (2004) 69–72. doi:10.1002/food.200300423.

[9] R. Arunkumar, K.V.H. Prashanth, V. Baskaran, Promising interaction between nanoencapsulated lutein with low molecular weight chitosan: Characterization and bioavailability of lutein in vitro and in vivo, Food Chem. 141 (2013) 327–337. doi:10.1016/j.foodchem.2013.02.108.

[10] M. Liu, J. Yang, P. Ao, C. Zhou, Preparation and characterization of chitosan hollow nanospheres for anticancer drug curcumin delivery, Mater. Lett. 150 (2015) 114–117. doi:10.1016/j.matlet.2015.03.013.

[11] S. Bharathiraja, N.Q. Bui, P. Manivasagan, M.S. Moorthy, S. Mondal, H. Seo, N.T. Phuoc, Multimodal tumor-homing chitosan oligosaccharide- coated biocompatible palladium nanoparticles for photo-based imaging and therapy, Sci. Rep. (2018) 1–16. doi:10.1038/s41598-017-18966-8.

[12] S. Maji, T. Agarwal, J. Das, T.K. Maiti, Development of gelatin / carboxymethyl chitosan / nano-hydroxyapatite composite 3D macroporous sca ff old for bone tissue engineering applications, Carbohydr. Polym. 189 (2018) 115–125. doi:10.1016/j.carbpol.2018.01.104.

[13] M. Rizzo, R.V. Giglio, D. Nikolic, A.M. Patti, C. Campanella, M. Cocchi, N. Katsiki, G. Montalto, Effects of chitosan on plasma lipids and lipoproteins: A 4-month prospective pilot study, Angiology. 65 (2014) 538–542. doi:10.1177/0003319713493126.

[14] E. Talón, K.T. Trifkovic, V.A. Nedovic, B.M. Bugarski, M. Vargas, A. Chiralt, C. González-Martínez, Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts, Carbohydr. Polym. 157 (2017) 1153–1161. doi:10.1016/j.carbpol.2016.10.080.

[15] J. Liang, H. Yan, X. Wang, Y. Zhou, X. Gao, P. Puligundla, X. Wan, Encapsulation of epigallocatechin gallate in zein/chitosan nanoparticles for controlled applications in food systems., Food Chem. 231 (2017) 19–24. doi:10.1016/j.foodchem.2017.02.106.

[16] J. Bonilla, P.J.A. Sobral, Investigation of the physicochemical, antimicrobial and antioxidant properties of gelatin-chitosan edible film mixed with plant ethanolic extracts, Food Biosci. 16 (2016) 17–25. doi:10.1016/j.fbio.2016.07.003.

[17] I. Leceta, S. Molinaro, P. Guerrero, J.P. Kerry, K. De la Caba, Quality attributes of map packaged ready-to-eat baby carrots by using chitosan-based coatings, Postharvest Biol. Technol. 100 (2015) 142–150. doi:10.1016/j.postharvbio.2014.09.022.

[18] E. Genskowsky, L.A. Puente, J.A. Pérez-Álvarez, J. Fernandez-Lopez, L.A. Muñoz, M. Viuda-Martos, Assessment of antibacterial and antioxidant properties of chitosan edible films incorporated with maqui berry (Aristotelia chilensis), LWT - Food Sci. Technol. 64 (2015) 1057–1062. doi:10.1016/j.lwt.2015.07.026.

[19] L. Sheng, M. Huang, J. Wang, Q. Xu, H.H.M. Hammad, M. Ma, A study of storage impact on ovalbumin structure of chicken egg, J. Food Eng. 219 (2018) 1–7. doi:10.1016/j.jfoodeng.2017.08.028.

[20] J.E. FOTHERGILL, L.A. FOTHERGILL, A.J.G. MOIR, A.D. NISBET, R.H. SAUNDRY, The Complete Amino-Acid Sequence of Hen Ovalbumin, Eur. J.

Biochem. 115 (2005) 335–345. doi:10.1111/j.1432-1033.1981.tb05243.x.

[21] P.P. Batra, K. Sasa, T. Ueki, K. Takeda, Circular Dichroic Study of Conformational Changes in Ovalbumin Prem P . Batra , 1 Katsushi Sasa , 2 Takuya Ueki , 2 and Kunio Takeda 2, 8 (1989) 221–229.

[22] N. Gharbi, M. Labbafi, Effect of processing on aggregation mechanism of egg white proteins, Food Chem. 252 (2018) 126–133. doi:10.1016/j.foodchem.2018.01.088.

[23] J.A. Huntington, P.E. Stein, Structure and properties of ovalbumin, J. Chromatogr. B Biomed. Sci. Appl. 756 (2001) 189–198. doi:10.1016/S0378-4347(01)00108-6.

[24] W. Xiong, C. Ren, M. Tian, X. Yang, J. Li, B. Li, Emulsion stability and dilatational viscoelasticity of ovalbumin/chitosan complexes at the oil-in-water interface, Food Chem. 252 (2018) 181–188. doi:10.1016/j.foodchem.2018.01.067.

[25] P.E. Stein, A.G.W. Leslie, J.T. Finch, R.W. Carrell, Crystal structure of uncleaved ovalbumin at 1·95 Å resolution, J. Mol. Biol. 221 (1991) 941–959. doi:10.1016/0022-2836(91)80185-W.

[26] (2017) Xiong, Z.; Ma, M - Enhanced ovalbumin stability at oil-water interface by phosphorylation and identification of phosphorylation site using MALDI-TOF mass spectrometry.pdf, (n.d.).

[27] Y. Chen, J. Hu, X. Yi, B. Ding, W. Sun, F. Yan, S. Wei, Z. Li, Interactions and emulsifying properties of ovalbumin with tannic acid, Lwt. 95 (2018) 282–288. doi:10.1016/j.lwt.2018.04.088.

[28] S. Sang, H. Zhang, L. Xu, Y. Chen, X. Xu, Z. Jin, N. Yang, F. Wu, D. Li, Functionality of ovalbumin during Chinese steamed bread-making processing, Food Chem. 253 (2018) 203–210. doi:10.1016/j.foodchem.2018.01.150.

[29] M.B. Santos, A.R. da Costa, E.E. Garcia-Rojas, Heteroprotein complex coacervates of ovalbumin and lysozyme: Formation and thermodynamic characterization, Int. J. Biol. Macromol. 106 (2018) 1323–1329. doi:10.1016/j.ijbiomac.2017.08.132.

[30] C.J.F. Souza, C.S.F. Souza, L.P. Heckert Bastos, E.E. Garcia-Rojas, Interpolymer complexation of egg white proteins and carrageenan: Phase behavior, thermodynamics and rheological properties, Int. J. Biol. Macromol. 109 (2018) 467–475. doi:10.1016/j.ijbiomac.2017.12.116.

[31] F.C. de Oliveira, J.S. dos R. Coimbra, E.B. de Oliveira, A.D.G. Zuñiga, E.E.G. Rojas, Food Protein-polysaccharide Conjugates Obtained via the Maillard Reaction: A Review, Crit. Rev. Food Sci. Nutr. 56 (2016) 1108–1125. doi:10.1080/10408398.2012.755669.

[32] Y.P. Timilsena, T.O. Akanbi, N. Khalid, B. Adhikari, C.J. Barrow, Complex coacervation: Principles, mechanisms and applications in microencapsulation, Int. J. Biol. Macromol. 121 (2019) 1276–1286. doi:10.1016/j.ijbiomac.2018.10.144.

[33] S.I.F.S. Martins, W.M.F. Jongen, M.A.J.S. Van Boekel, A review of Maillard reaction in food and implications to kinetic modelling, 11 (2001) 364–373.

[34] F. Comert, P.L. Dubin, Liquid-liquid and liquid-solid phase separation in protein-polyelectrolyte systems, Adv. Colloid Interface Sci. 239 (2017) 213–217. doi:10.1016/j.cis.2016.08.005.

[35] J. Pathak, E. Priyadarshini, K. Rawat, H.B. Bohidar, Complex coacervation in charge complementary biopolymers: Electrostatic versus surface patch binding, Adv. Colloid Interface Sci. 250 (2017) 40–53. doi:10.1016/j.cis.2017.10.006.

[36] C. Schmitt, S.L. Turgeon, Protein/polysaccharide complexes and coacervates in food systems., Adv. Colloid Interface Sci. 167 (2011) 63–70. doi:10.1016/j.cis.2010.10.001.

[37] C. Liang, F. Yuan, F. Liu, Y. Wang, Y. Gao, Structure and antimicrobial mechanism of ε-polylysine-chitosan conjugates through Maillard reaction, Int. J. Biol. Macromol. 70 (2014) 427–434. doi:10.1016/j.ijbiomac.2014.07.012.

[38] Q. Liu, B. Kong, J. Han, C. Sun, P. Li, Structure and antioxidant activity of whey protein isolate conjugated with glucose via the Maillard reaction under dry-heating conditions, Food Struct. 1 (2014) 145–154. doi:10.1016/j.foostr.2013.11.004.

[39] J. Feng, S. Wu, H. Wang, S. Liu, Improved bioavailability of curcumin in ovalbumin-dextran nanogels prepared by Maillard reaction, J. Funct. Foods. 27 (2016) 55–68. doi:10.1016/j.jff.2016.09.002.

[40] K. Li, M.W. Woo, H. Patel, C. Selomulya, Enhancing the stability of protein-polysaccharides emulsions via Maillard reaction for better oil encapsulation in spray-dried powders by pH adjustment, Food Hydrocoll. 69 (2017) 121–131. doi:10.1016/j.foodhyd.2017.01.031.

[41] S. Pirestani, A. Nasirpour, J. Keramat, S. Desobry, J. Jasniewski, Effect of glycosylation with gum Arabic by Maillard reaction in a liquid system on the emulsifying properties of canola protein isolate, Carbohydr. Polym. 157 (2017) 1620–1627. doi:10.1016/j.carbpol.2016.11.044.

[42] C. Hou, S. Wu, Y. Xia, G.O. Phillips, S.W. Cui, A novel emulsifier prepared from Acacia seyal polysaccharide through Maillard reaction with casein peptides, Food Hydrocoll. 69 (2017) 236–241. doi:10.1016/j.foodhyd.2017.01.038.

[43] Y. Liu, M.J. Selig, M.P. Yadav, L. Yin, A. Abbaspourrad, Transglutaminase-treated

conjugation of sodium caseinate and corn fiber gum hydrolysate: Interfacial and dilatational properties, Carbohydr. Polym. 187 (2018) 26–34. doi:10.1016/j.carbpol.2018.01.034.

[44] S. Xia, O. Mukeshimana, I. Habinshuti, X. Xu, B. Muhoza, E. Duhoranimana, J. Yu, E. Karangwa, X. Zhang, Thermodynamic characterization of Gelatin–Sodium carboxymethyl cellulose complex coacervation encapsulating Conjugated Linoleic Acid (CLA), Food Hydrocoll. 80 (2018) 149–159. doi:10.1016/j.foodhyd.2018.02.011.

[45] H. Chen, A. Ji, S. Qiu, Y. Liu, Q. Zhu, L. Yin, Covalent conjugation of bovine serum album and sugar beet pectin through Maillard reaction/laccase catalysis to improve the emulsifying properties, Food Hydrocoll. 76 (2018) 173–183. doi:10.1016/j.foodhyd.2016.12.004.

[46] L. Consoli, R.A.O. Dias, R.S. Rabelo, G.F. Furtado, A. Sussulini, R.L. Cunha, M.D. Hubinger, Sodium caseinate-corn starch hydrolysates conjugates obtained through the Maillard reaction as stabilizing agents in resveratrol-loaded emulsions, Food Hydrocoll. 84 (2018) 458–472. doi:10.1016/j.foodhyd.2018.06.017.

[47] M.M. Baracat, A.M. Nakagawa, R. Casagrande, S.R. Georgetti, W.A. Verri, O. de Freitas, Preparation and Characterization of Microcapsules Based on Biodegradable Polymers: Pectin/Casein Complex for Controlled Drug Release Systems, AAPS PharmSciTech. 13 (2012) 364–372. doi:10.1208/s12249-012-9752-0.

[48] M.A. Azevedo, A.I. Bourbon, A.A. Vicente, M.A. Cerqueira, Alginate/chitosan nanoparticles for encapsulation and controlled release of vitamin B 2, Int. J. Biol. Macromol. 71 (2014) 141–146. doi:10.1016/j.ijbiomac.2014.05.036.

[49] H. Ilyasoglu, S.N. El, Nanoencapsulation of EPA/DHA with sodium caseinate-gum arabic complex and its usage in the enrichment of fruit juice, LWT - Food Sci. Technol. 56 (2014) 461–468. doi:10.1016/j.lwt.2013.12.002.

[50] F. Diarrassouba, G. Remondetto, G. Garrait, P. Alvarez, E. Beyssac, M. Subirade, Self-assembly of β-lactoglobulin and egg white lysozyme as a potential carrier for nutraceuticals, Food Chem. 173 (2015) 203–209. doi:10.1016/j.foodchem.2014.10.009.

[51] T.A. Comunian, J. Gomez-Estaca, R. Ferro-Furtado, G.J.A. Conceição, I.C.F. Moraes, I.A. De Castro, C.S. Favaro-Trindade, Effect of different polysaccharides and crosslinkers on echium oil microcapsules, Carbohydr. Polym. 150 (2016) 319–329. doi:10.1016/j.carbpol.2016.05.044.

[52] A.L. Chapeau, G.M. Tavares, P. Hamon, T. Croguennec, D. Poncelet, S. Bouhallab, Spontaneous co-assembly of lactoferrin and β-lactoglobulin as a promising biocarrier

for vitamin B9, Food Hydrocoll. 57 (2016) 280–290. doi:10.1016/j.foodhyd.2016.02.003.

[53] A. Jain, D. Thakur, G. Ghoshal, O.P. Katare, U.S. Shivhare, Characterization of microcapsulated β-carotene formed by complex coacervation using casein and gum tragacanth, Int. J. Biol. Macromol. 87 (2016) 101–113. doi:10.1016/j.ijbiomac.2016.01.117.

[54] Y. Yuan, Z.Y. Kong, Y.E. Sun, Q.Z. Zeng, X.Q. Yang, Complex coacervation of soy protein with chitosan: Constructing antioxidant microcapsule for algal oil delivery, Lwt. 75 (2017) 171–179. doi:10.1016/j.lwt.2016.08.045.

[55] J. Gomez-Estaca, T.A. Comunian, P. Montero, C.S. Favaro-Trindade, Physico-Chemical Properties, Stability, and Potential Food Applications of Shrimp Lipid Extract Encapsulated by Complex Coacervation, Food Bioprocess Technol. (2018) 1–9. doi:10.1007/s11947-018-2116-3.

[56] L. Tavares, C.P. Zapata Noreña, Encapsulation of garlic extract using complex coacervation with whey protein isolate and chitosan as wall materials followed by spray drying, Food Hydrocoll. 89 (2019) 360–369. doi:10.1016/j.foodhyd.2018.10.052.

[57] S. Yu, J. Hu, X. Pan, P. Yao, M. Jiang, Stable and pH-sensitive nanogels prepared by self-assembly of chitosan and ovalbumin, Langmuir. 22 (2006) 2754–2759. doi:10.1021/la053158b.

[58] W. Xiong, C. Ren, W. Jin, J. Tian, Y. Wang, B.R. Shah, J. Li, B. Li, Ovalbumin-chitosan complex coacervation: Phase behavior, thermodynamic and rheological properties, Food Hydrocoll. 61 (2016) 895–902. doi:10.1016/j.foodhyd.2016.07.018.

[59] K. Wang, D. Sun, H. Pu, Q. Wei, Trends in Food Science & Technology Principles and applications of spectroscopic techniques for evaluating food protein conformational changes : A review, Trends Food Sci. Technol. 67 (2017) 207–219. doi:10.1016/j.tifs.2017.06.015.

[60] T. Edition, Principles of Fluorescence Spectroscopy, n.d.

[61] M.C.Æ.A. Nucara, Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region, (2010) 679–690. doi:10.1007/s00726-009-0274-3.

[62] S.E. Glassford, B. Byrne, S.G. Kazarian, Biochimica et Biophysica Acta Recent applications of ATR FTIR spectroscopy and imaging to proteins, BBA - Proteins Proteomics. 1834 (2013) 2849–2858. doi:10.1016/j.bbapap.2013.07.015.

[63] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism,

Biochim. Biophys. Acta - Proteins Proteomics. 1751 (2005) 119–139. doi:10.1016/j.bbapap.2005.06.005.

CHAPTER TWO

MANUSCRIPT:

A MULTI-SPECTROSCOPIC STUDY OF CHITOSAN-OVALBUMIN SUPRAMOLECULAR STRUCTURES FORMED AT DIFFERENT PHS

A multi-spectroscopic study of chitosan-ovalbumin supramolecular structures formed at different pHs

Gustavo Leite Milião [a,*], Lucas de Souza Soares [a], Éverton de Almeida Alves Barbosa [b], Douglas Fernando Balbino [a], Gustavo Costa Bressan [b], Alvaro Vianna Novaes de Carvalho Teixeira [c], Jane Sélia dos Reis Coimbra [a], Eduardo Basílio de Oliveira [a,*].

[a] *Departamento de Tecnologia de Alimentos (DTA), Universidade Federal de Viçosa (UFV), Campus Universitário, 36570-900 Viçosa, MG, Brazil*

[b] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa (UFV), Campus Universitário, 36570-900 Viçosa, MG, Brazil*

[c] *Departamento de Física, Universidade Federal de Viçosa (UFV), Campus Universitário, 36570-900 Viçosa, MG, Brazil*

[*] Corresponding authors: gustavo.miliao@ufv.br; eduardo.basilio@ufv.br

Abstract

Ovalbumin (OVA) and chitosan (CHS) interaction in aqueous solution of chloride acid, at different pH values (4.0 and 6.0), was studied in order to evaluate if the presence of the polysaccharide would cause any expressive conformational changes in the overall protein structure. Then, a multi-spectroscopic approach, using UV-Vis and fluorescence to analyze the tertiary structure of OVA, conjointly with FT-IR and circular dichroism to study possible secondary structure modifications, was undertaken. UV-Vis and fluorescence results showed that the biomolecules interacted at some molecular level, forming complexes without a major modification in the microenvironment polarity of the protein. Furthermore, the fluorescence analysis suggested that the quenching mechanism of chitosan is likely static, since the bimolecular quenching constant k_q value (1.21 x 10^{13} $L \cdot mol^{-1} \cdot s^{-1}$) is greater than the maximum diffusion collision quenching constant of macromolecules (2.00 x 10^{10} $L \cdot mol^{-1} \cdot s^{-1}$). Moreover, CD and FT-IR spectroscopies revealed that changes in the secondary structure of OVA upon interacting with CHS depended on the concentration of the polysaccharide. This study showed that the biomolecules could interact at both pH values forming complexes that could be applied in food, pharmaceutical, and medical applications.

Keywords: protein, polysaccharide, interaction, spectroscopies, supramolecular structures.

Abbreviations and symbols

CC – complex coacervation

CD – circular Dichroism

CHS – chitosan

deg - degrees

DA – Degree of Acetylation

DD – Degree of Deacetylation

F_o – fluorescence intensity before chitosan addition

F – fluorescence intensity after chitosan addition

FT-IR – Fourier Transform Infrared

H-bonding – hydrogen bonding

k_q – bimolecular quenching constant

K_{sv} – Stern-Volmer constant

M_w – molecular weight

OVA – ovalbumin

O/W – oil in water

pH – hydrogenionic potential

Phe - phenylalanine

SS – supramolecular structure

Trp – tryptophan

Tyr – tyrosine

UV-Vis – UV-Visible

θ – mean residue ellipticity

1. INTRODUCTION

The study of protein-polysaccharide interactions has been attracting attention of both academic and industrial fields, owing to the fact that the supramolecular structures (SS) obtained from the coupling of such biopolymers may be applied mainly to encapsulate bioactive compounds [1-4] or to improve the physical stability and texture properties of formulated colloidal systems, such as emulsions and foams [5,6], for the cosmetic, pharmaceutical and food industries. These two classes of biomacromolecules may associate through covalent binding, forming conjugates via the Maillard reaction, or non-covalent interactions, which include electrostatic, hydrophobic, H-bonding, and van der Waals interactions [7,8], promoting the formation of complexes/coacervates.

The importance of different protein-polysaccharide interactions has been frequently reported in literature [9-13], along with the wide technological applicability of the obtained structures. However, to the best of our knowledge, few studies about two specific biopolymers – the protein ovalbumin (OVA) and the polysaccharide chitosan (CHS) – have been described for food applications so far. Ovalbumin is the main constituent protein in egg white, accounting for approximately 55% of the total protein content [14]. Besides its use in standard preparation for the investigation of protein structures and properties as well as in experimental models of allergy, OVA shows itself as a potential emulsifier due to its hydrophobicity and net electric charge [15]. On the other hand, chitosan, which is obtained from deacetylation of chitin, is a random block copolymer composed of glucosamine and N-acetyl-glucosamine, linked by β-(1–4)-glycosidic bonds. The degree of acetylation (DA) of chitosan, which corresponds to the percentage of N-acetyl glucosamine, along with the average molecular weight (M_w) of the macromolecule, constitutes the two most relevant of its physicochemical features. In addition to the fact that chitosan comes from the second most abundant polysaccharide (chitin), it is a biodegradable, biocompatible, and non-toxic biopolymer that presents a unique characteristic of becoming positive at acidic media (pH < 6,4) and, therefore, it has shown wide applicability in medical, pharmaceutical, wastewater treatment, tissue engineering, and food industries [16]. Although some papers have reported the use of systems containing OVA and CHS for pharmaceutical and medical purposes [17,18], the application of such systems specifically for the food industry was not directly addressed in the studies found. Furthermore, the literature lacks a deeper molecular investigation of how ovalbumin-chitosan interaction, at different pH values, may influence the protein physicochemical properties and, consequently, its techno-functionality.

Two studies about supramolecular structures formed from OVA-CHS interaction have been described so far. Xiong et al. [19] studied the phase separation kinetics, thermodynamics and rheological aspects of OVA-CHS interaction (at pH 4.0 and 5.5). In this case, the systems were characterized in terms of turbidity, zeta potential, thermodynamic parameters of interaction, microstructure, and rheological properties. Results showed that at both pH the phenomenon of complexation was spontaneous and exothermic, and the rheological properties of the systems depended on the medium ionic strength and OVA:CHS ratio. At ionic strength less than 50 $mmol \cdot L^{-1}$ and OVA:CHS mass ratios ranging from 1:1 to 3:1, the coacervates presented stronger gels than those with higher salt concentrations (> 50 $mmol \cdot L^{-1}$) and protein:polysaccharide ratios varying from 3:1 to 10:1. In another study, Xiong et al. [15] evaluated the effect of OVA-CHS supramolecular structures as O/W emulsion stabilizers (at pH 4.0 and 5.5), through rheological properties of such model emulsion systems and interfacial dilatational properties of the complexes adsorbed at the oil-water interface. Results showed that the viscoelastic and interfacial properties of the emulsions and complexes, respectively, improved when OVA-CHS structures were added to the system, at pH 5.5. The authors attributed this effect to the low mobility of emulsion droplets and the increase of the layer thickness around the droplets, which conferred higher stability to the colloidal system when compared to emulsions stabilized by OVA alone.

Such studies have introduced the importance of evaluating OVA-CHS complexes with potential applicability in the food sector. However, they did not report nor discussed the conformational changes of ovalbumin upon the SS formation with chitosan in aqueous solution of HCl with different pH values, through a multi-spectroscopic approach. We believe that an understanding of these aspects could be a key-point to design specific applications of such systems in food formulations, but also to widen their applicability in the pharmaceutical and medical sectors. However, it is necessary to know how the interaction could affect the physicochemical features of the biomolecules in order to address the complexes to technological applications. Therefore, the objective of this study was to evaluate if OVA and CHS could interact at a molecular level and at different pH values (4.0 and 6.0), as well as to assess protein conformational features, through a multi-spectroscopic approach, namely as UV-Vis, Fourier Transform Infrared (FT-IR), circular dichroism (CD), and intrinsic fluorescence spectroscopies. Aside from the reports about ovalbumin and chitosan interaction, our study is the first one which seeks to understand the association of such biopolymers in a molecular level, with a set of distinct spectroscopic techniques, at two different pH values and, therefore, it hopefully will bring new information on what have been observed in literature so far.

2. MATERIALS AND METHODS

2.1. Materials

Ovalbumin (OVA, A5503, 98% pure by agarose gel electrophoresis, isoelectric point (pI) at pH ~ 4.9, and a molecular weight of 44.3 kDa) from *Gallus gallus domesticus* was purchased from Sigma Co. (Saint Louis – MO, USA). Before usage, OVA was dialyzed, according to the methodology adopted by Galván et al. [20], in order to assure the removal of residual low molecular solutes (mainly electrolytes) that could interfere in further experiments. Subsequently, the dialyzed protein was lyophilized (LS 3000, Terroni, Brazil) and packed under vacuum at -18 °C (vertical freezer, GE, FZGE320, Brazil) until usage in all subsequent experiments. Chitosan (CHS, low molecular weight (~162.0 kDa – data not shown); Degree of Deacetylation: 80.3%; pKa ~ 6.4; Product ID: 448869; Batch number: SLBG1673V) from *Pandallus borealis*, a cold water shrimp, was also obtained from Sigma-Aldrich (Saint Louis – MO, USA). Prior to its use, CHS was sequentially washed three times with deionized water, aiming at eliminating impurities, such as salt residues and water soluble chitooligosaccharides. Then, the degree of deacetylation (DD) of chitosan was estimated (~77.0% - data not shown) using an FT-IR spectrometer (600-IR, Varian, USA) equipped with an attenuated reflectance accessory (GladiATR, PIKE Technologies, USA) over the region of (450-4000 cm^{-1}), as described by Soares et al. [21], who adapted the methodologies from Kasaai [22] and Brugnerotto et al. [23]. The slight difference from the DD provided by Sigma may be attributed to the washing step that might have removed compounds of small molecular mass that would contribute to increasing DD of chitosan. All other chemicals, such as chloride acid (HCl P.A. ACS; Code: 1353), sodium hydroxide, and sodium azide were also purchased from Sigma-Aldrich (Saint Louis – MO, USA). In all experiments, deionized water (QUV3, 69 Millipore, Italy; electrical resistivity ~ 18.2 $M\Omega \cdot cm^{-1}$ at 25 °C) was used.

2.2. Preparation of OVA and CHS dispersions

Two OVA stock dispersions (0.20%, m/v), were prepared by dispersing the protein powder in chloride acid (HCl) aqueous solution (pH 4.0 or 6.0). The dispersions were stirred gently (300 rpm) with a magnetic stirrer, at room temperature, for 1 h, and then they were kept under agitation overnight, at 4 °C, to ensure protein rehydration. Likewise, two CHS dispersions (0.20%, m/v), were obtained by adding the polysaccharide powder in chloride acid aqueous solution (pH 4.0 or 6.0) under intensive magnetic stirring (1000 rpm) overnight, at 25 °C. The pH of both resulting dispersions was adjusted to 4.0 or 6.0, by the addition of HCl (1.0 $mol \cdot L^{-}$

[1]) or NaOH (1.0 mol·L^{-1}). Moreover, sodium azide (0.01%, w/v) was added in all dispersions in order to inhibit the growth of microorganisms.

2.3. Spectroscopic approaches

2.3.1. UV-Vis absorption spectroscopy

The UV-vis absorption spectra were obtained for both pH values (4.0 and 6.0) in a UV-1800 spectrophotometer (Shimadzu, Japan), using a quartz cuvette of 1 cm path length. All of them were recorded in the wavelength range of 200-400 nm, at 25 ± 1 °C. OVA concentration was fixed at 5.0 μmol·L^{-1}, whereas CHS concentration varied from 0.0 μmol·L^{-1} (control) to 4.0 μmol·L^{-1}. Also, the spectrum of CHS (4.0 μmol·L^{-1}) alone was measured. All UV-Vis spectra were corrected by subtracting the proper baseline. Peaks formed close to the wavelength range 270-280 nm are related to the microenvironment polarity around the aromatic amino acids tryptophan and tyrosine. Therefore, in order to evaluate complexes formation and possible structural changes on the protein upon interacting with chitosan, shifts in the maximum absorption peak and spectra intensity increase were evaluated.

2.3.2. Fluorescence emission spectroscopy

The fluorescence analyses of OVA dispersions (pH 4.0 or 6.0) at a fixed concentration of 20.0 μmol·L^{-1} in the presence of several CHS concentrations (0.0, 3.0, 4.0, 5.0 and 6.0 μmol·L^{-1}) were performed in a K2 spectrofluorimeter (ISS, USA), using a quartz cuvette (Suprasil®) of 10 mm path length. The emission spectra were collected at 25 ± 1 °C, controlled by a circulating water bath 9001 (PolyScience, USA). The excitation wavelength was set at 280 nm, in order to only excite tryptophan and tyrosine residues, and the emission wavelength range was between 290-450 nm. The excitation and emission slit widths were fixed at 5 nm. All spectra were corrected by subtracting the proper baseline.

Additionally, in order to evaluate the fluorescence quenching mechanism in the OVA, the Stern-Volmer equation (1) [24-26] was applied:

$$\frac{F_o}{F} = 1 + K_{sv} \cdot [CHS] = 1 + k_q \tau_o \cdot [CHS] \qquad \text{(Eq. 1)}$$

in Eq. (1), F_o and F are the fluorescence intensities before and after the addition of the polysaccharide, respectively; K_{sv} is the Stern-Vomer constant; $[CHS]$ is the concentration of the quencher; k_q is the bimolecular quenching constant; and τ_o is the lifetime of the fluorophore in the absence of the quencher (~10^{-8} s) [9].

2.3.3. Fourier Transform – Infrared (FT-IR) spectroscopy

FT-IR spectroscopy was applied in order to analyze the biopolymer dispersions at both pH values (4.0 and 6.0), at 25 ± 1 °C. Prior to the analyses, the protein with a fixed concentration (5.0 μmol·L^{-1}) was mixed with different CHS concentrations (0.0 μmol·L^{-1}, control, to 4.0 μmol·L^{-1}). Subsequently, these systems (protein alone and mixtures) were lyophilized (LS 3000, Terroni, Brazil). The analyses were carried out directly on the lyophilized systems, that is, the OVA powder (alone) and the mixture containing different amounts of the polysaccharide, using a spectrometer (600-IR, Varian, USA) equipped with an attenuated reflectance accessory (GladiATR, PIKE Technologies, USA) over the region of (1200-1700 cm^{-1}). The absorption spectra were normalized and deconvolved in Lorentzian components in the PeakFit software (v.4.12, SeaSolve Software Inc., 1999-2003). Each spectrum was an average of three measurements. Protein and peptides infrared spectra give rise to five typical bands, being three of them the amide bands (I, II, and III), which are the most common used to study protein structure. These three amide bands correspond to the wavenumber region range of 1200-1700 cm^{-1}. The spectra from such regions are mainly due to stretching vibrations of C=O coupled with C–N stretch, and N–H bending [27,28] and because of this, it is the often used to verify protein secondary structure modifications. Therefore, the analyses were performed in the wavenumber region of 1200-1700 cm^{-1}, in order to evaluate possible changes.

2.3.4. Circular Dichroism (CD) spectroscopy

CD spectra of the protein dispersion, at pH 4.0 and 6.0, were obtained in a Jasco J-810 spectropolarimeter (Jasco Corporation, Japan) fitted with a temperature controller Peltier PFD 425S (Jasco Corporation, Japan) and coupled to a thermostated bath AWC 100 (Julabo, Germany). OVA concentration was fixed at 5.0 μmol·L^{-1}, whereas CHS concentration varied from 0.0 μmol·L^{-1} (control) to 3.0 μmol·L^{-1}. All CD spectra were collected at 25 ± 1 °C (unless it is stated otherwise), under nitrogen flush to avoid equipment damage, using a 0.1 cm path length quartz cell, in the far-UV wavelength range of 200-260 nm and a data pitch of 1.0 nm. The scanning speed, bandwidth, and slit width were set to 100 nm·min^{-1}, 1.0 nm, and 1.0 μm, respectively. Ten accumulations were taken in order to improve the signal to noise ratio of the experiment. Prior to the measurements, the OVA stock dispersions had to be diluted at least 8 fold. All CD spectra were corrected by subtracting the proper baseline and normalized to protein mean molar residue ellipticity [θ_{OVA}] was normalized according to the Equation 1.

$$[\theta_{OVA}] = \frac{\theta \cdot 100 \cdot MM}{C_{OVA} \cdot n \cdot l} \qquad \text{Eq. 1}$$

in which, $[\theta_{OVA}]$ is the ovalbumin mean molar residue ellipticity, θ is the CD signal (mdeg) obtained in the spectropolarimeter, MM is the protein molar mass (kDa), n is the number of amino acid residues, C_{OVA} is the ovalbumin concentration ($mg \cdot mL^{-1}$) and l is the optical path of the cuvette. Additionally, the secondary structure content in the presence and absence of CHS were estimated using the CONTIN analysis program with the support of DICHROWEB (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [24,29].

3. RESULTS

The interaction between OVA and CHS was evaluated through a multi-spectroscopic approach in order to evaluate if supramolecular structures would be formed and consequently if conformational changes in the protein structure would happen. Such characterization is important to amplify the complexes/coacervates application in the food, medical and pharmaceutical industries, since the SS obtained usually presents better techno-functional and biofunctional properties than the biomacromolecules individually [7,9]. Figure 1 presents the UV-Vis, FT-IR, CD, and fluorescence spectra of the native protein (without CHS) and the complexes (OVA in the presence of different CHS concentrations) at pH 4.0 and 6.0. The results of each spectroscopic analysis are reported in sections 3.1 to 3.4. followed by the discussion in the section 4.

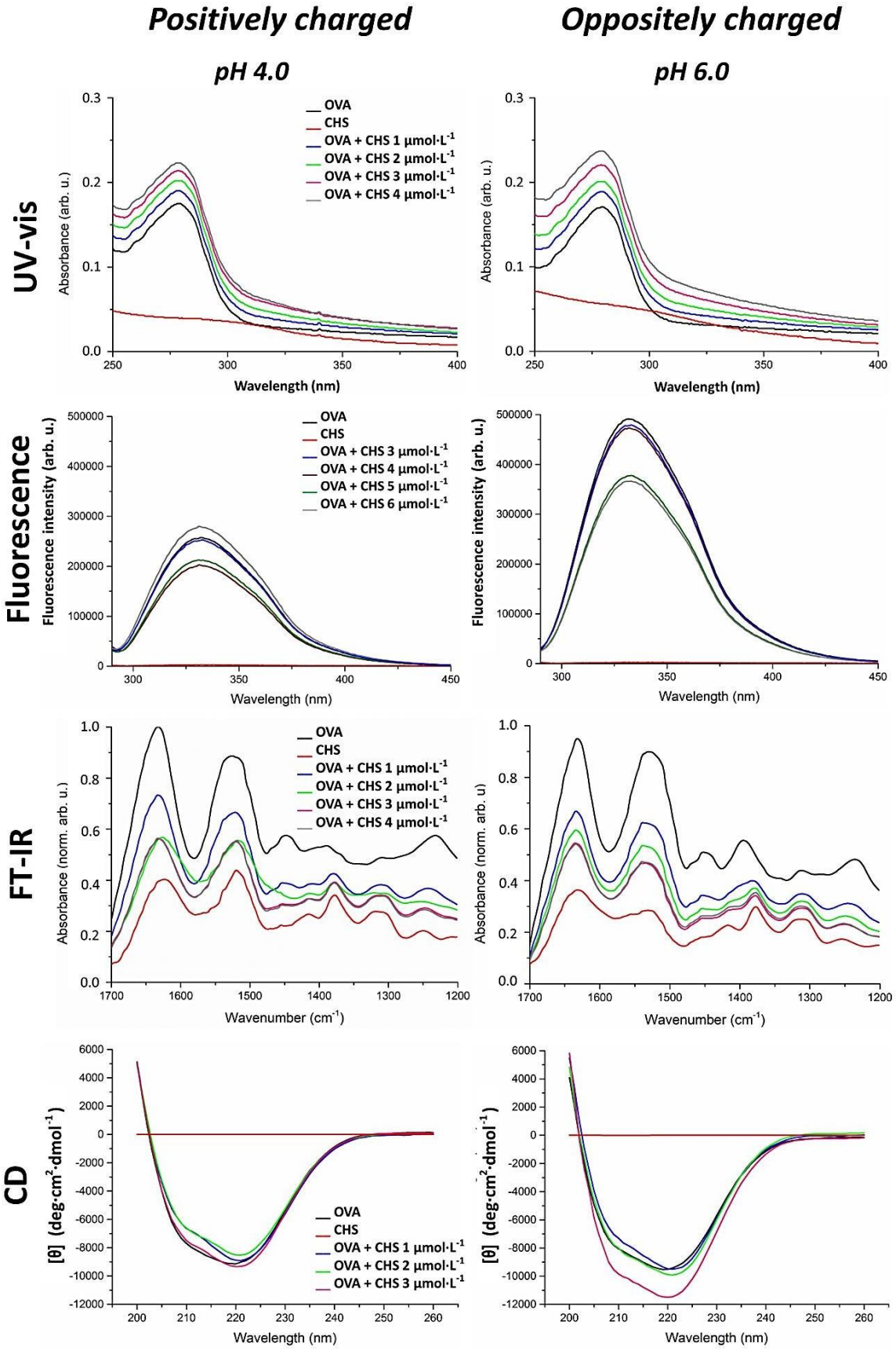


Figure 1 – Absorption and emission spectra of OVA in the presence of different CHS concentrations at pH 4,0 (macromolecules are positively charged) and pH 6,0 (macromolecules are oppositely charged).

3.1. UV-Vis absorption spectroscopy

UV-Vis spectroscopy was used to qualitatively evaluate the formation of complexes between OVA and CHS at both pH values (4.0 and 6.0) and, therefore, possible alterations in the tertiary structure of the protein by means of the microenvironment change around the chromophore groups, which are the aromatic aminoacid residues [30].

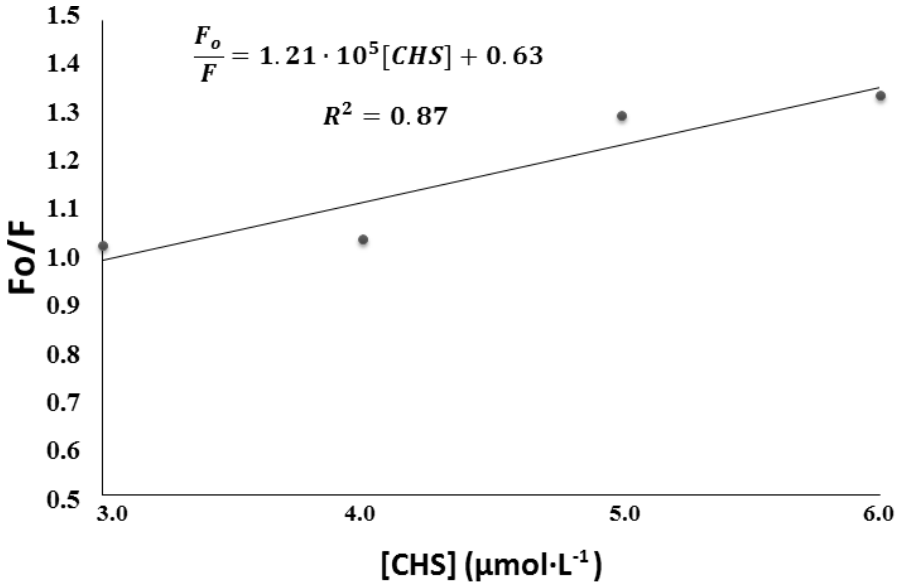
As depicted in Fig. 1, the maximum peak of each spectrum occurred at 278 nm. Furthermore, the higher the chitosan concentration, the greater was the UV-Vis absorption intensity of the systems. In terms of microenvironment, polarity around tryptophan and tyrosine, no shift in the maximum UV-Vis absorption peaks was observed with the increase of CHS. These behaviors were observed for both pH values 4.0 and 6.0.

3.2. Intrinsic fluorescence spectroscopy

OVA is a glycoprotein with three tryptophan and nine tyrosine residues, that in contact with the microenvironment surrounding them could provide information about possible conformational changes in the structure of the biomacromolecule [11,25]. Fig. 1 shows the emission spectra of OVA (excited at 280 nm) in the absence and presence of CHS, at pH 4.0 and 6.0. The spectrum of the protein alone is characterized by a single peak at around 331 and 333 nm, for pH 6.0 and 4.0, respectively.

In terms of fluorescence intensity, two behaviors were observed for each pH value. At pH 4.0, smaller concentrations of chitosan (3.0, 4.0, and 5.0 $\mu mol \cdot L^{-1}$) quenched the fluorescence emission of the protein, whereas the largest CHS concentration (6.0 $\mu mol \cdot L^{-1}$) led to a slight increase on the emission. On the other hand, at pH 6.0, it is noticeable that as CHS concentration increased from 3.0 $\mu mol \cdot L^{-1}$ to 6.0 $\mu mol \cdot L^{-1}$ the fluorescence intensity of OVA was quenched. For both pH values, no obvious shift of OVA emission spectra was observed. Since the quenching phenomenon was only clearly noticed at pH 6.0, the Stern-Volmer equation (1) was applied to estimate the K_{sv}.

Figure 2 shows a plot of $\frac{F0}{F}$ versus $[CHS]$, in which the slope of the curve yielded the Stern-Volmer constant. Noticeably, the k_q value (1.21 x 10^{13} $L \cdot mol^{-1} \cdot s^{-1}$) is greater than the maximum diffusion collision quenching constant of macromolecules (2.00 x 10^{10} $L \cdot mol^{-1} \cdot s^{-1}$) [9].



	K_{sv} $(L \cdot mol^{-1})$	$k_q(L \cdot mol^{-1} \cdot s^{-1})$	R^2
pH 6.0	**$1.21 \cdot 10^5$**	**$1.21 \cdot 10^{13}$**	**0.87**

Figure 2 - Stern-Volmer plot of OVA with increasing CHS concentrations.

3.3. Fourier Transform – Infrared (FT-IR) spectroscopy

The structural characterization of OVA (5.0 µmol·L^{-1}) at different CHS concentrations (0.0 to 4.0 µmol·L^{-1}) was evaluated by FT-IR spectroscopy, which gives rise to spectra from vibrational motions, mainly stretching and bending, of chemical bonds. As already stated in literature, in proteins, the three most prominent bands for structural characterization are: amide I near 1,650 cm^{-1} (C=O stretch conjointly with weak C–N stretch and N–H bend), amide II near 1,550 cm^{-1} (N–H bend and C–N stretch), and amide III near 1,300 cm^{-1} (C–N stretch, N–H bend, along with deformation vibrations of C–H and N–H) [27,28]. For both pH values, changes in the intensity and bands displacement were regarded as possible secondary conformational alteration.

At pH 6.0, native OVA (5.0 µmol·L^{-1}) spectrum presented a slight peak at 1,654 cm^{-1} and a stronger one at 1,631 cm^{-1} (C=O stretching) in the amide I band. Moreover, in the amide II band, other three characteristic peaks were observed at 1,566, 1,541, and 1,513 cm^{-1} (C–N stretch and N–H bend). For the amide III, the bands were found to be at 1,311, 1,273, and 1,234 cm^{-1} (C–N stretch and N–H bend). Upon adding CHS at different concentrations, no major displacement was noticed for the amide band I (1,654 to 1,656 cm^{-1}). On the other hand, for amide band II, the most expressive changes observed were shifts from 1,566 to 1,588 cm^{-1} and

from 1,513 to 1,520 cm^{-1} (C–N stretch and N–H bend), at CHS concentration of 3.0 $\mu mol \cdot L^{-1}$. Regarding amide band III, spectra shift was noticed, at CHS 1.0 $\mu mol \cdot L^{-1}$, from 1,311 to 1,301 cm^{-1} and from 1,234 to 1,251 (C–N stretch and N–H bend).

Similarly, at pH 4.0, native OVA (5.0 $\mu mol \cdot L^{-1}$) spectrum showed two peaks in the amide I region at 1,659 and 1,627 cm^{-1} (C=O stretching), which presented no expressive band shift upon polysaccharide addition (1,655 and 1,630 cm^{-1}, respectively). For amide II band, another peak was observed at 1,511 cm^{-1} with a shift band to 1,517 cm^{-1}, when CHS concentration was 1.0 $\mu mol \cdot L^{-1}$ (C–N stretch and N–H bend). Furthermore, the amide III band showed two characteristic peaks at: 1,311 (native protein) with band shifts to 1,308, 1,319, and 1,323 cm^{-1} for CHS concentrations of 1.0, 2.0, and 4.0 $\mu mol \cdot L^{-1}$ (C–N stretch and N–H bend), respectively; and 1,252 cm^{-1} (native protein) with band shifts to 1,239, 1,242, and 1,247 cm^{-1} for CHS concentrations of 1.0, 2.0, and 4.0 $\mu mol \cdot L^{-1}$ (C–N stretch and N–H bend), respectively. Noticeably, in terms of absorbance, as CHS concentration increased the intensity of absorption reduced, for both pH values.

3.4. Circular Dichroism (CD) spectroscopy

Circular dichroism is a spectroscopic technique which allows an assessment of proteins conformational changes, upon the interaction with ligands or submitted to different extrinsic medium conditions. Theoretically, at wavelengths below 260 nm (far UV), the absorption of light is mostly because of the peptide bond, in which there is a broad $n \rightarrow \pi^*$ transition (at ~ 220 nm) and an even more intense $\pi \rightarrow \pi^*$ transition near to 190 nm. On the other hand, at wavelengths above 260 nm (near UV) the spectra arise mostly because of the aromatic aminoacids [31]. Therefore, depending on the region of study, modifications in both secondary and tertiary structures may be evaluated. In this study, the spectra of OVA, in the absence and in the presence of CHS (1.0-3.0 $\mu mol \cdot L^{-1}$), were recorded in the range of 200-260 nm, in order to evaluate if there were any changes in the secondary structure of the protein with the increase in chitosan concentration. CD spectrum of native ovalbumin, at pH 4.0 and 6.0, presented negative peaks in the range between 208 and 222 nm (Fig. 1), which is typical of α- helical regions.

However, at pH 6.0, a quantitative analysis of the spectra revealed that native OVA (without CHS) had approximately 4.2% α-helix, 34.5% β-sheet, 4.6% β-turn, and 56.8% random coil structures. At 1.0 $\mu mol \cdot L^{-1}$ and 2.0 $\mu mol \cdot L^{-1}$ CHS concentrations, the changes in α-helix content ranged from 4.2% (native) to 4.6% and 5.5%, respectively. Furthermore, the molar ellipticity value slightly decreased. On the other hand, at the same CHS concentrations,

a minor increase in β-sheet content (34.5% to 39.9 and 38.8%, respectively) concomitantly with a decrease in random coil (56.8% to 55.6 and 37.5%, respectively) was observed. Additionally, at 3.0 μmol·L^{-1} CHS, it was noticeable an expressive raise in the molar ellipticity value of OVA, when compared to the other concentrations. In this case, α-helix and β-turn content increased from 4.2% and 4.6% (native) to 15.2%, whereas β-sheet and random coil structures decreased from 34.5% and 56.8% to 13.7% and 55.9%, respectively. No major shift (in terms of blue or red shift) was observed in the spectra after the addition of CHS.

At pH 4.0, the spectra deconvolution yielded analogous results (to pH 6.0) for the native protein (OVA without CHS), which contained 4.2% α-helix, 36.2% β-sheet, 4.6% β-turn, and 55.0% random coil. Furthermore, at 1.0 μmol·L^{-1} of CHS an increase in α-helix (6.3%) and β-sheet (42.9%) simultaneously with a decrease in β-turn (0.0%) and random coil (50.7%) was observed. On the other hand, when adding 2.0 μmol·L^{-1} and 3.0 μmol·L^{-1} of CHS did not trigger substantial changes in the protein secondary structure. Moreover, no expressive shift in the spectra was observed upon CHS addition. Table 1 summarizes the percentage of each secondary structure of OVA, at pH 4.0 and 6.0.

Table 1 - Percentages of different elements of the secondary structure of OVA in the absence and presence of CHS (1.0 - 3.0 μmol·L^{-1}), at pH 4.0 and 6.0 (T = 25 °C).

pH 4.0 (both macromolecules are positively charged)				
Sample	% (α-helix)	% (β-sheet)	% (β-turn)	% (random coil)
OVA without CHS	4.2	36.2	4.6	55.0
OVA-CHS 1.0 μmol·L^{-1}	6.3	42.9	0.0	50.7
OVA-CHS 2.0 μmol·L^{-1}	4.9	38.5	3.6	53.0
OVA-CHS 3.0 μmol·L^{-1}	4.4	39.2	0.0	56.5
pH 6.0 (macromolecules are oppositely charged)				
OVA without CHS	4.2	34.5	4.6	56.8
OVA-CHS 1.0 μmol·L^{-1}	4.6	39.9	0.0	55.6
OVA-CHS 2.0 μmol·L^{-1}	5.5	38.3	18.6	37.5
OVA-CHS 3.0 μmol·L^{-1}	15.2	13.7	15.2	55.9

4. DISCUSSION

In order to understand the nature of the intermolecular interactions between OVA and CHS as well as if possible structural changes occured in the protein, four distinct spectroscopies were undertaken: UV-Vis, Fourier transform-infrared, circular dichroism, and intrinsic

fluorescence. In this section, we aimed at relating the results that were observed in each spectroscopy to gain insights and propose hypothesis on how the association between the macromolecules would happen.

Even though UV-Vis spectroscopy is widely used to studyconformational changes of proteins when in contact with ligands, it gives only qualitative information and should be applied conjointly with other techniques. Therefore, the OVA-CHS complex formation was monitored through UV-Vis absorption and fluorescence emission, in order to assess tertiary conformational modifications on OVA.

In UV-Vis spectra, the appearance of peaks at 278 nm originates from the $\pi \rightarrow \pi^*$ transition of the aromatic residues [mainly tyrosine (Tyr) and tryptophan (Trp)]. In its native conformation ovalbumin presents three Trp and nine Tyr residues which are highly sensitive to the microenvironment where they are inserted. Slight perturbations around these aminoacids residues could suggest that some sort of interaction would be happening between the biomacromolecules, yielding possible changes in the structure of the protein.

For pH 6.0, the higher the concentration of CHS, the greater was the UV-Vis absorption intensity of the systems. Other studies have reported the same behavior for different biopolymers interactions, namely konjac glucomannan–*Zanthoxylum armatum* DC. seed glutelin [9], ovalbumin–gum arabic [11], and procyanidin dimer–α-amylase [26]. Furthermore, regarding the fluorescence spectroscopy, the augment in polysaccharide concentration caused the quenching phenomena of the protein, indicating that OVA and CHS interacted at some level. It is also noteworthy that, in terms of microenvironment polarity, no shift in the maximum UV-Vis absorption and fluorescence emission peaks was observed with the increase of CHS, which is an indicative of no major tertiary structure change of the protein.

Fluorescence quenching, i.e., the decrease in fluorescence intensity of a sample, may be a result of several intermolecular interactions [25] and it appears as an outstanding spectroscopic approach to study the interaction between proteins and several ligands. Tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) are the major intrinsic fluorophores present in proteins. However, Trp, which is highly sensitive to the microenvironment where it is inserted, exerts the maximum effect on protein fluorescence due to the indole group and, therefore, it might be used to locate this residue in polypeptide chains. As it can be seen in Fig. 1, the spectrum of the protein alone, at pH 6.0 is characterized by a single peak at around 331 nm, which is typical of well-folded proteins, with intrinsic fluorophores buried within the hydrophobic pocket [25,32]. Indeed, Fig. 3 shows that the Trp residues are located in the inner part of the polypeptide chain (in the native OVA conformation). Since at this pH CHS and OVA

are oppositely charged, electrostatic interactions between $-NH_3^+$ groups (present on CHS backbone) and $-COO^-$ (present on the protein) were expected, being the driving force of complex formation and quenching of the fluorescence. However, hydrogen bond, hydrophobic interactions, and van der Waals forces, may also be stabilizing the structure of OVA, as it was observed by CD and FT-IR spectroscopies.

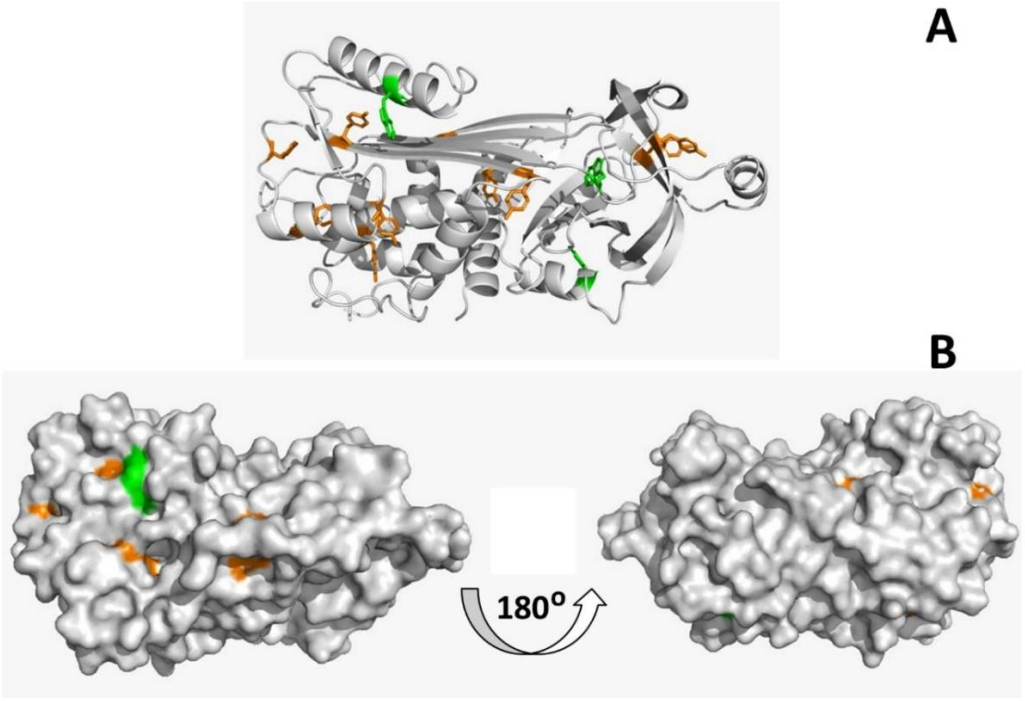


Figure 3 – Ribbon structure (A) and Connolly surface structure of the OVA (B). Green and orange colors represent the tryptophan and tyrosine residues, respectively. These images were elaborated with data from the PDB entry 1OVA [33], without any modification of the crystal atomic coordinates, using (Pymol, version 2.2) software package.

According to Lakowicz [33], fluorescence quenching may be either static or dynamic (collisional), depending on the type of molecular contact between the protein and the ligand. Static quenching is characterized by the formation of a non-fluorescent complex between the fluorophores in the ground state and the quencher. In turn, for the collisional quenching to happen, the quencher has to diffuse onto the fluorophore during the lifetime of the excited state. At pH 6.0, in which the macromolecules were oppositely charged, the k_q value ($1.21 \cdot 10^{13}$ $L \cdot mol^{-1} \cdot s^{-1}$) showed to be about one thousand greater than the maximum diffusion collision quenching constant commonly admitted for macromolecules ($2.00 \cdot 10^{10}$ $L \cdot mol^{-1} \cdot s^{-1}$) [14], suggesting that the mechanism of fluorescence quenching between OVA and CHS would be static. At pH 4.0 (both biopolymers positively charged), such constant could not be calculated and, therefore, no inference could be done about the mechanisms of interaction between the biomolecules. In order to better clarify the quenching mechanism of OVA by CHS, at pH 6.0, the behavior of K_{sv} as a function of temperature should be further evaluated.

Similar behavior was noticed for the interaction between the biopolymers at pH 4.0. The maximum emission peak of OVA (without CHS) was around 333 nm, indicating that the polypeptide chain was well-folded, with the intrinsic fluorophores inside the protein structure. Moreover, Fig. 1 shows that with the increase in CHS concentration from 3.0 $\mu mol \cdot L^{-1}$ to 5.0 $\mu mol \cdot L^{-1}$, the emission spectra of OVA were progressively quenched. Besides, the increasing of CHS concentration [0.0 to 4.0 $\mu mol \cdot L^{-1}$] promoted the raise in the UV-Vis absorption of protein. Another noteworthy behavior was noticed for CHS 6.0 $\mu mol \cdot L^{-1}$, in which the fluorescence intensity increased. We believe that at this highest chitosan concentration, the polysaccharide could have affected the protein structure in a way that the fluorophores would be slightly exposed to the solvent, explaining the increase in the fluorescence intensity. These conjoint spectroscopic results indicate that OVA and CHS interacted at some molecular level, especially through non-electrostatic interaction types, since both biomolecules are positively charged, without a major wavelength shift and consequently a significant conformational modification in the protein tertiary structure. Such idea can also be corroborated by the FT-IR results, which showed that at a given concentration shift bands occurred mostly due to C–N stretch and N–H bend, suggesting that hydrogen bonding and van der Waals interactions could be promoting the supramolecular structures formation.

At both pH values, the quenching phenomenon could be attributed to the fact that inter and intramolecular interactions among the protein and polysaccharide chains occurred, inhibiting the fluorescence emission of the intrinsic fluorophores that would be surrounded by chains of the macromolecules. In addition, chitosan is a polysaccharide which presents $-NH_3^+$ groups (at pH < pKa = 6.4) that along with other electron-deficient groups, such as protonated histidine, lysine, and –COOH may provoke the quenching of the Trp residues [25]. We hypothesize that this effect could occur due to the capability of such electron-deficient groups to difficult the emission of excited electrons on the molecules. Additionally, at pH 4.0, OVA is close to its isoelectric point (pH 4.9), possibly presenting weak intermolecular electrostatic repulsion and, consequently, enabling the interaction of some chains of the protein, which, in turn, would favor the fluorescence quenching, due to the hiding of some intrinsic fluorophores. Noticeably, the fluorescence intensity of OVA at this pH is much weaker when compared to that at pH 6.0. Niu et al. [11] observed the same quenching phenomenon when studying the interaction between ovalbumin and gum Arabic at pH 4.0 and 6.0. Likewise, they attributed the quenching phenomenon to the collision of gum Arabic chains and protein molecules, which would cause perturbations around the Trp residues, forcing this aminoacid to move to the hydrophobic pocket of the protein.

Regarding the CD spectrum of native ovalbumin at pH 6.0, two negative peaks appeared in the range between 208 nm and 222 nm (Fig. 1), typically of α-helical proteins. Upon spectra deconvolution, native OVA (without CHS) showed approximately 4.2% α-helix, 34.5% β-sheet, 4.6% β-turn, and 56.8% random coil structures (Table 1). Such result corroborates the fact that proteins with high fractions of β-sheet generally exhibit greater denaturation temperatures than those which contain large amount of α-helix structures [34]. Indeed, OVA presents denaturation temperature around 84 °C, that could be explained by the greater thermic stability of β-sheet when compared to α-helix. Jesus et al. [17], similar to what we have observed, found that native OVA presented nearly 35% β-sheet, when they studied the impact of chitosan inclusion in poly-ε-caprolactone nanoparticles on the secondary structure of adsorbed ovalbumin, using CD in the region of 190-260 nm.

Noticeably, at 1.0 $\mu mol \cdot L^{-1}$ and 2.0 $\mu mol \cdot L^{-1}$ CHS concentrations, the changes in α-helix content was negligible, ranging from 4.2% (native) to 4.6% and 5.5%, respectively. Furthermore, the molar ellipticity value slightly decreased at these concentrations, suggesting that the interaction of the polysaccharide to the protein did not cause a major modification on this type of secondary structure of OVA. On the other hand, at the same CHS concentrations, the increase in β-sheet content (34.5% to 39.9 and 38.8%, respectively) concomitantly with a decrease in random coil (56.8% to 55.6 and 37.5%, respectively) would indicate that these kinds of secondary structures could give rise to a stabilization mechanism of OVA, in the CHS presence, through hydrogen bonding between the carbonyl groups on the protein and the amino groups on the polysaccharide and protein molecules. Indeed, as it is well known, β-sheet structures are mostly stabilized by H-bonding, which corroborates our hypothesis. FT-IR results also support such possible protein conformational change as it interacts with chitosan, since a spectra shift in the amide III band was noticed from 1,311 to 1,301 cm^{-1} and 1,234 to 1,251 (C–N stretch and N–H bend) when in the presence of CHS 1.0 $\mu mol \cdot L^{-1}$.

Additionally, at 3.0 $\mu mol \cdot L^{-1}$ CHS, the conjoint analyses of CD and FT-IR, showed the increase in the molar ellipticity value and the spectral shift in the amide II band (C–N stretch and N–H bend), respectively. It suggests that the macromolecules interacted, causing major secondary structure modifications on the protein, when compared to the other concentrations. Moreover, it was noticeable that at all chitosan concentrations studied, FT-IR spectra intensities diminished, indicating that CHS interacted with C=O, N–H, and C–N groups present on OVA [35] most likely through electrostatic attraction (biopolymers were oppositely charged), hydrogen bonds, and van der Waals interactions.

A possible explanation for these behaviors is that, at pH 6.0, the polysaccharide is close to its pKa value (6.4) and, therefore, the content of $–NH_3^+$ in the chitosan chains is approximately 55.0%. In this case, no much intra and/or intermolecular repulsion would be happening, which would cause the chains of the polysaccharide to be closer to each other, forming agglomerated 'packs' of CHS. As a consequence, some positively charged patches on CHS could be unavailable to interact with the predominantly negative charged protein and other types of interaction rather than electrostatic (hydrophobic, H-bonding, and van der Waals) could be happening in a lesser extent, especially with the increase in chitosan concentration. In general, these results indicate that the interaction between the biopolymers somehow affects the structure of the protein mostly when in the presence of higher polysaccharide concentrations (3.0 $\mu mol \cdot L^{-1}$).

A complementary circular dichroism study of ovalbumin in the absence and presence of chitosan at pH 4.0, in which both biopolymers would be positively charged, was performed, in order to verify if conformational modifications in the protein occurred. At 2.0 $\mu mol \cdot L^{-1}$ and 3.0 $\mu mol \cdot L^{-1}$ CHS, no major modification in the content of secondary structures was perceived by CD spectroscopy. However, FT-IR results showed that the presence of the polysaccharide at higher concentrations (mostly 3.0 and 4.0 $\mu mol \cdot L^{-1}$) caused the bands to shift, indicating that the interaction between the biopolymers somehow affected the overall secondary structure of the protein. In addition, at 1.0 $\mu mol \cdot L^{-1}$ CHS, there was an increase in β-sheet content simultaneously with a decrease in random coil quantity, suggesting that the interaction of OVA with CHS may have stabilized the β-structure [17]. FT-IR analyses also showed that, at this concentration, there was a slight shift of amide II band (C–N stretch and N–H bend), confirming that interactions between the macromolecules may have occurred. The major modification on the protein structure at the lowest CHS concentration could be a consequence of less charged patches on chitosan backbone, which would decrease the electrostatic repulsion between the macromolecules, enabling other types of interactions, such as hydrophobic, hydrogen bonding, and van der Waals to happen. However, a more detailed thermodynamic study through isothermal titration calorimetry (ITC) and intrinsic fluorescence at different temperatures, for instance, would be necessary to confirm this hypothesis. Such techniques provide thermodynamic parameters (ΔH, ΔS and ΔG), which allows the inference about the interaction type between molecules. In addition, molecular modelling could be a powerful tool to address the specific intermolecular interactions that would be taking place between the biomolecules. Therefore, as a future perspective, our research team intends to perform *in silico* studies on the

OVA and CHS association, in order to shed a light on the intermolecular interaction between these biopolymers.

5. CONCLUSION

The interaction between ovalbumin and chitosan at different pH values (4.0 and 6.0) were evaluated through a multi-spectroscopic approach aiming at understanding if conformational changes would happen in the protein. At both pH values, we noticed that upon increasing CHS concentration, the UV-Vis absorption spectra of OVA augmented, whereas the emission fluorescence intensity was quenched, revealing the interaction between the biomacromolecules. However, since no spectra shift was clearly observed, it was assumed that the protein tertiary structure had no major modification. In addition, CD and FT-IR spectroscopies were used to investigate possible modifications on the protein secondary structure in the presence of CHS. Results showed that slight changes occurred, which could be an indicative of OVA-CHS supramolecular formation through different types of interactions, at pH 4.0 and 6.0. In addition to the molecular phenomena involving OVA/CHS interactions discussed in the present study, *in silico* molecular modeling studies are now on the way in our lab, aiming at find the specific OVA interaction sites between with CHS, as well as identifying the nature of such interactions.

Acknowledgements

The authors are deeply grateful to: Brazilian research agencies CNPq, CAPES (Finance Code 001), and FAPEMIG, for their financial support; Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), for allowing us to use some facilities in the Laboratório Nacional de Biociências (LNBio); *D. Sc*. Eber A. A. Medeiros and Prof. Nilda de Fátima F. Soares, who permitted us to use the laboratory supervised by them; *M. Sc*. Thomás Valente de Oliveira, for his kind help in designing the 3D molecular structure of ovalbumin; and Mr. Márcio Alvarenga, for his technical support in performing the FT-IR analyses.

References

[1] R. Shaddel, J. Hesari, S. Azadmard-Damirchi, H. Hamishehkar, B. Fathi-Achachlouei, Q. Huang, Use of gelatin and gum Arabic for encapsulation of black raspberry anthocyanins by complex coacervation. Int. J. Biol. Macromol. 107 (2018) 1800-1810.

[2] H. Xie, C. Xiang, Y. Li, L. Wang, Y. Zhang, Z. Song, X. MA, X. Lu, Q. Lei, W. Fang, Fabrication of ovalbumin/κ-carrageenan complex nanoparticles as a novel carrier for curcumin delivery, Food Hydrocolloid. 89 (2019) 111-121.

[3] A. Jain, D. Thakur, G. Ghoshal, O. P. Katare, U. S. Shivare, Characterization of microcapsulated β-carotene formed by complex coacervation using casein and gum tragacanth. Int. J. Biol. Macromol. 87 (2016) 101-113.

[4] X. Ren, T. Hou, Q. Liang, D. Zhang, D. Hu, B. Xu, X. Chen, M. Chalamaiah, H. Ma, Effects of frequency ultrasound on the properties of zein-chitosan complex coacervation for resveratrol encapsulation, Food Chem. 279 (2019) 223-230.

[5] Q. Zhao, Z. Long, J. Kong, T. Liu, D. Sun-Waterhouse, M. Zhao, Sodium caseinate/flaxseed gum interactions at oil–water interface: Effect on protein adsorption and functions in oil-in-water emulsion, Food Hydrocolloid. 43 (2015) 137-145.

[6] A. A. Perez, C. R. Carrara, C. C. Sánchez, L. G. Santiago, J. M. R. Patino, Interfacial dynamic properties of whey protein concentrate/polysaccharide mixtures at neutral pH, Food Hydrocolloid. 23 (2009) 1253-1262.

[7] M. Semenova, Protein–polysaccharide associative interactions in the design of tailor-made colloidal particles, Curr. Opin. Colloid Interface Sci. 28 (2017) 15-21.

[8] A. K. Ghosh, P. Bandyopadhyay, Polysaccharide-protein interactions and their relevance in food colloids. In: The complex world of polysaccharides, InTech. 1 ed. (2012). http://dx.doi.org/10.5772/2947.

[9] Y. Qu, X. Hou, J. Ke, G. Shen, M. Ye, Z. Zhang, Mechanistic investigation on binding interaction of konjac glucomannan with *Zanthoxylum armatum* DC. seed glutelin in solution, Int. J. Biol. Macromol. 123 (2019), 308-313.

[10] C. J. F. Souza, C. S. F. Souza, L. P. H. Bastos, E. E. Garcia-Rojas, Interpolymer complexation of egg white proteins and carrageenan: Phase behavior, thermodynamics and rheological properties, Int. J. Biol. Macromol. 109 (2018) 467–475.

[11] F. Niu, Y. Dong, F. Shen, J. Wang, Y. Liu, Y. Su, R. Xu, J. Wang, Y. Yang, Ovalbumin–gum arabic interactions: Effect of pH, temperature, salt, biopolymers ratio and total concentration. Colloid Surface B, v. 113, p. 477-482, 2014.

[12] S. F. Mirpoor, S. M. H. Hosseini, G. H. Yousefi, Mixed biopolymer nanocomplexes conferred physicochemical stability and sustained release behavior to introduced curcumin, Food Hydrocolloid. 71 (2017) 216-224.

[13] E. Duhoranimana, J. Yu, O. Mukeshimana, I. Haabinshuti, E. Karangwa, X. Xu, B. Muhoza, S. Xia, Thermodynamic characterization of Gelatine-Sodium carboxymethylcellulose complex coacervation encapsulating Conjugated Linoleic Acid (CLA), Food Hydrocolloid. 80 (2018) 149-159.

[14] L. Sheng, M. Huang, J. Wang, Q. Xu, H. H. M. Hammad, M. Ma, A study of storage impact on ovalbumin structure of chicken egg, J. Food Eng. 219 (2018) 1-7.

[15] W. Xiong, C. Ren, M. Tian, X. Yang, J. Li, B. Li, Emulsion stability and dilatational viscoelasticity of ovalbumin/chitosan complexes at the oil-in-water interface, Food Chem. 252 (2018) 181–188.

[16] V. Zargar, M. Asghari, A. Dashti, A review on chitin and chitosan polymers: structure, chemistry, solubility, derivatives, and applications, Chembioeng Reviews. v. 2, n. 3 (2015) 204-226.

[17] S. Jesus, E. H. Fragal, A. F. Rubira, E. C. Muniz, A. J. M. Valente, O. Borges, The Inclusion of Chitosan in Poly-ε-caprolactone Nanoparticles: Impact on the Delivery System Characteristics and on the Adsorbed Ovalbumin Secondary Structure, AAPS PharmSciTech, 19 (2018) 101-113.

[18] Y. Ishii-Mizuno, Y. Umeki, Y. Onuki, H. Watanabe, Y. Takahashi, Y. Takakura, M. Nishikawa, Improved sustained release of antigen from immunostimulatory DNA hydrogel by electrostatic interaction with chitosan, Int. J. Pharm. 516 (2017) 392-400.

[19] W. Xiong, C. Ren, W. Jin, J. Tian, Y. Wang, B. R. Shah, J. Li, B. Li, Ovalbumin-chitosan complex coacervation: Phase behavior, thermodynamic and rheological properties, Food Hydrocolloid. 61 (2016) 895-902.

[20] Z. R. N. Galván, L. S. Soares, E. A. A. Medeiros, N. F. F. Soares, A. M. Ramos, J. S. R. Coimbra, E.B. Oliveira, Rheological properties of aqueous dispersions of xanthan gum containing different chloride salts are impacted by both sizes and net electric charges of the cations, Food Biophys. 13 (2018) 186-197.

[21] L. S. Soares, R. B. Perim, E. S. de Alvarenga, L. M. Guimarães, A. V. N. C. Teixeira, J. S. R. Coimbra, E. B. Oliveira, Insights on physicochemical aspects of chitosan dispersion in aqueous solutions of acetic, glycolic, propionic or lactic acid, Int. J. Biol. Macromol. 128 (2019) 140-148.

[22] M. R. Kasaai, A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy, Carbohydr. Polym. 71 (2008) 497-508.

[23] J. Brugnerotto, J. Lizardi, F.M. Goycoolea, W. Argülles-Monal, J. Desbrières, M. Rinaudo, An infrared investigation in relation with chitin and chitosan characterization Polym., 42 (2001) 3569-3580.

[24] J. Cheng, J. H. Liu, G. Prasanna, P. Jing, Spectrofluorimetric and molecular docking studies on the interaction of cyanidin-3-O-glucoside with whey protein, β-lactoglobulin, Int. J. Biol. Macromol. 105 (2017) 965-972.

[25] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 3, Springer US, 2006, http://dx.doi.org/10.1007/978-0-387-46312-4, XXVI, 954.

[26] T. Dai, J. Chen, Q. Li, P. Li, P. Hu, C. Liu, T. Li, Investigation the interaction between procyanidin dimer and α-amylase: Spectroscopic analyses and molecular docking simulation, Int. J. Biol. Macromol 113 (2018) 427-433.

[27] S. E. Glassford, B. Byrne, S.G. Kazarian, Recent applications of ATR FT-IR spectroscopy and imaging to proteins. BBA-Proteins Proteom. 1834 (2013) 2849-2858.

[28] M. Carbonaro, A. Nucara, Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region, Amino acids. 38 (2010) 679-690.

[29] X. Xu, W. Liu, J. Zhong, L. Luo, C. Liu, S. Luo, L. Chen, Binding interaction between rice glutelin and amylose: Hydrophobic interaction and conformational changes, Int. J. Biol. Macromol. 81 (2015) 942-950.

[30] K. Wang, D. W. Sun, H. Pu, Q. Wei, Principles and applications of spectroscopic techniques for evaluating food protein conformational changes: A review, Trends Food Sci. Technol. 67 (2017) 207-219.

[31] S. M. Kelly, T. J. Jess, N. C. Price, How to study proteins by circular dichroism, BBA-Proteins Proteom. 1751 (2005) 119-139.

[32] H. K. S. Souza, M. P. Gonçalves, J. Gómez, Effect of chitosan degradation on its interaction with β-lactoglobulin, Biomacromolecules, 12 (2011) 1015-1023.

[33] P. E. Stein, A. G. W. Leslie, J. T. Finch, R. W. Carrel, Crystal structure of uncleaved ovalbumin at 1· 95 Å resolution. Journal of molecular biology, v. 221, n. 3, p. 941-959, 1991.

[34] S. Damodaran, Amino Acids, Peptides, and Proteins, In: S. Damodaran, K. L. Parkin, O. R. Fennema (Ed.), Fennema's Food Chemistry, 4th ed., CRC Press, Boca Raton, (2007) 217-330.

[35] L. Bekale, D. Agudelo, H. A. Tajmir-Riahi, Effect of polymer molecular weight on chitosan–protein interaction, Colloid Surface B, 125 (2015) 309-317.

GENERAL CONCLUSION

Protein-polysaccharide supramolecular structures have demonstrated to be a strategic alternative to be used in technological applications, due to their improved techno-functionality as food additives (emulsifying, gelling and stabilizing agents) when compared to the biopolymers native form. Furthermore, it is noteworthy their wide applicabity as bioactive carriers to enrich formulations.

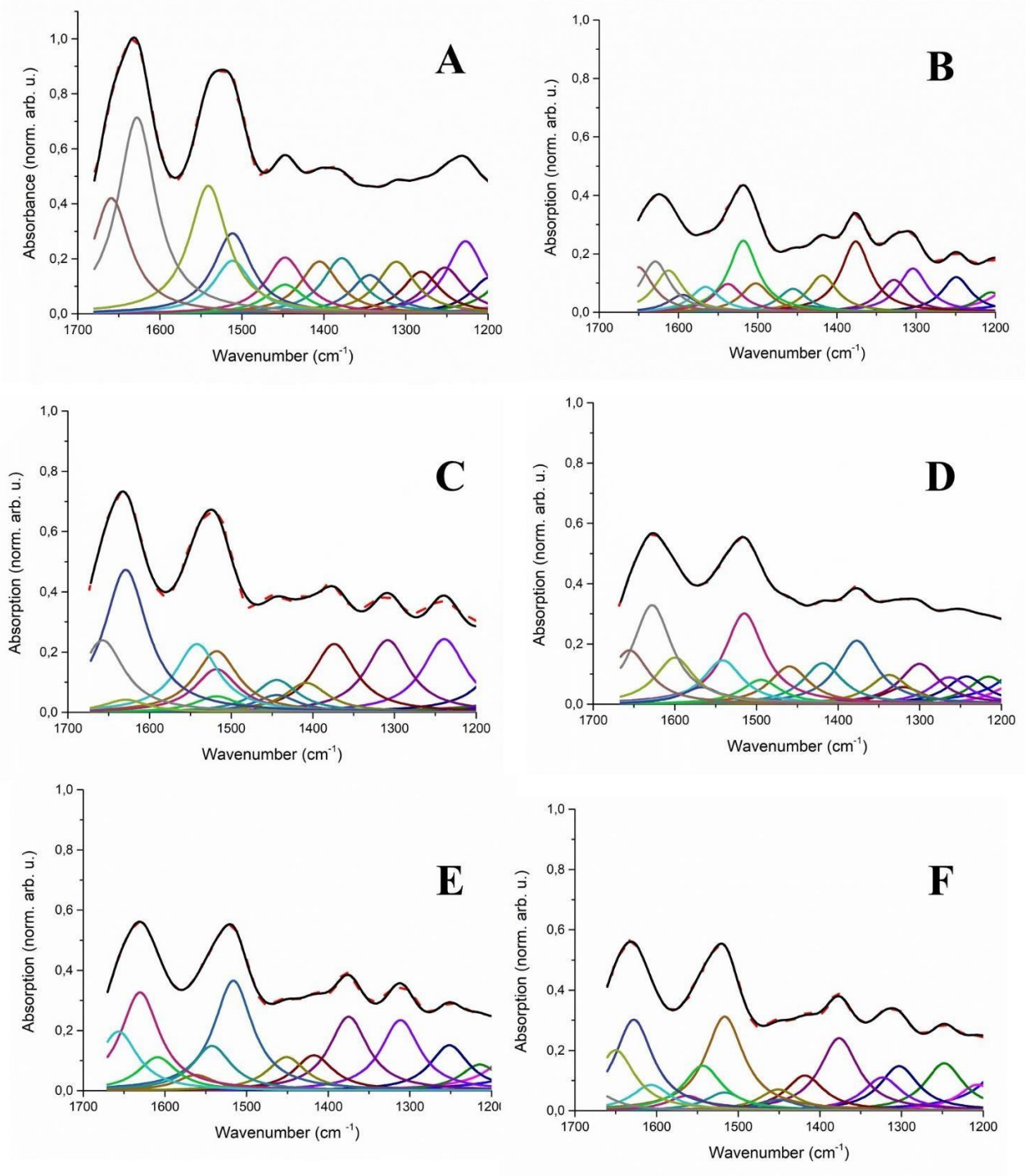
In this present work, the study about the interaction between two biopolymers commonly used in the food industry, the protein ovalbumin and the polysaccharide chitosan, was performed. Both macromolecules are biocompatible, biodegradable and non-toxic materials which makes them suitable for a variety of applications. However, in order to explore the bio/techno-functionality of the complexes obtained from the interaction between these biopolymers, it is needed to understand the mechnisms behind the association, so it is possible to direct the structures to the right application.

Therefore, our research team sought, for the first time, to understand, in a molecular basis and through a multi-spectroscopic approach, the formation of supramolecular structures from OVA-CHS interaction. Such research allowed the comprehension of basic and advanced science, which contributed to the researchers' professional and (most important) human resource development. Besides, it provided to the student abilities to develop strategic ways to better plan his activities as well as to solve problems. Moreover, by performing this project, the student was capable of dealing with several challenges, such as financial limitation, equipment unavailability and, consequently, results obtention. Nonetheless, it was possible to gather a reasonable set of results that allowed us to prepare this document. Hopefully, the information generated in this dissertation will be used as a reference to other studies in this related area.

As a perspective, we intend to keep studying the exactly mechanisms of interaction between OVA and CHS through more sophisticated techniques, that will allow our research group to wide the technological applicability of OVA-CHS supramolecular structures.

Appendix

A.1. FT-IR spectra of (A) OVA [5.0 µmol·L^{-1} (control)] (B) CHS [4.0 µmol·L^{-1} (control)] (C) OVA-CHS 1.0 µmol·L^{-1} (D) OVA-CHS 2.0 µmol·L^{-1} (E) OVA-CHS 3.0 µmol·L^{-1} (F) OVA-CHS 4.0 µmol·L^{-1}. Absorbance from FT-IR (--) through deconvolution in Lorentzian components (—), at pH 4.0. The protein concentration was fixed (5.0 µmol·L^{-1}) in the mixtures.



A.2. FT-IR spectra of (A) OVA [5.0 µmol·L^{-1} (control)] (B) CHS [4.0 µmol·L^{-1} (control)] (C) OVA-CHS 1.0 µmol·L^{-1} (D) OVA-CHS 2.0 µmol·L^{-1} (E) OVA-CHS 3.0 µmol·L^{-1} (F) OVA-CHS 4.0 µmol·L^{-1}. Absorbance from FT-IR (--) through deconvolution in Lorentzian components (—), at pH 6.0. The protein concentration was fixed (5.0 µmol·L^{-1}) in the mixtures.